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(71) Applicant: DOW CHEMICAL (AUSTRALIA) LIMITED [AU/AU]; Forest Corporate Park, 26 Rodborough Road, Frenchs Forest, NSW 2086 (AU).

(72) Inventors: MEZES, Peter, S. ; 5101 Oakridge Drive, Midland, MI 48640 (US). RICHARD, Ruth, A. ; 5111 Plainfield Street, Midland, MI 48642 (US). JOHNSON, Kim, S. ; 3765 Freedom Court, Midland, MI 49642 (US).

(74) Agent: SPRUSON & FERGUSON; GPO Box 3898, Sydney, NSW 2001 (AU).

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(54) Title: COMPOSITE ANTIBODIES OF HUMAN SUBGROUP IV LIGHT CHAIN CAPABLE OF BINDING TO TAG-72

(57) Abstract

This invention concerns a subset of composite Hum4 V_L, $V_H\alpha$TAG antibody with high affinities to a high molecular weight, tumor-associated sialylated glycoprotein antigen (TAG-72) of human origin. These antibodies have variable regions with (1) V_L segments derived from the human subgroup IV germline gene and (2) a V_H segment which is capable of combining with the V_L to form a three dimensional structure having the ability to bind TAG-72. *in vivo* methods of treatment and diagnostic assay using these composite antibodies is also disclosed.

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COMPOSITE ANTIBODIES OF HUMAN SUBGROUP IV LIGHT CHAIN CAPABLE OF BINDING TO TAG-72

The present invention is directed to the fields of immunology and genetic engineering.

The following information is provided for the purpose of making known information believed by the applicants to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the following information constitutes prior art against the present invention.

Antibodies are specific immunoglobulin (Ig) polypeptides produced by the vertebrate immune system in response to challenges by foreign proteins, glycoproteins, cells, or other antigenic foreign substances. The binding specificity of such polypeptides to a particular antigen is highly refined, with each antibody being almost exclusively directed to the particular antigen which elicited it.

Two major methods of generating vertebrate antibodies are presently utilized:generation *in situ* by the mammalian B lymphocytes and generation in cell culture by B-cell hybrids. Antibodies are generated *in*

situ as a result of the differentiation of immature B lymphocytes into plasma cells (see Gough (1981), Trends in Biochem Sci, 6:203 (1981). Even when only a single antigen is introduced into the immune system for a particular mammal, a uniform population of antibodies does not result, i.e., the response is polyclonal.

The limited but inherent heterogeneity of polyclonal antibodies is overcome by the use of hybridoma technology to create "monoclonal" antibodies in cell cultures by B cell hybridomas (see Kohler and Milstein (1975). Nature. 256:495-497). In this process, a mammal is injected with an antigen, and its relatively short-lived, or mortal, splenocytes or lymphocytes are fused with an immortal tumor cell line. The fusion produces hybrid cells or "hybridomas" which are both immortal and capable of producing the genetically-coded antibody of the B cell.

In many applications, the use of monoclonal antibodies produced in non-human animals is severely restricted where the monoclonal antibodies are to be used in humans. Repeated injections in humans of a "foreign" antibody, such as a mouse antibody, may lead to harmful hypersensitivity reactions, i.e., an anti-idiotypic, or human anti-mouse antibody (HAMA) response, (see Shawler *et al.* (1985), Journal of Immunology, 135:1530-1535. and Sear *et al.*, J. Biol. Resp. Modifiers, 3:138-150).

Various attempts have already been made to manufacture human-derived monoclonal antibodies by using human hybridomas (see Olsson *et al.*, Proc. Natl. Acad. Sci. U.S.A., 77:5429 (1980) and Roder *et al.* (1986), Methods in Enzymology, 121:140-167. Unfortunately,

yields of monoclonal antibodies from human hybridoma cell lines are relatively low compared to mouse hybridomas. In addition, human cell lines expressing immunoglobulins are relatively unstable compared to mouse cell lines, and the antibody producing capability of these human cell lines is transient. Thus, while human immunoglobulins are highly desirable, human hybridoma techniques have not yet reached the stage where human monoclonal antibodies with required antigenic specificities can be easily obtained.

Thus, antibodies of nonhuman origin have been genetically engineered, or "humanized". Humanized antibodies reduce the HAMA response compared to that expected after injection of a human patient with a mouse antibody. Humanization of antibodies derived from nonhumans, for example, has taken two principal forms, i.e., chimerization where non-human regions of immunoglobulin constant sequences are replaced by corresponding human ones (see for example, USP 4,816,567 to Cabilly *et al.*, Genentech) and grafting of complementarity determining regions (CDR) into human framework regions (FR) (see European Patent Office Application (EPO) 0 239 400 to Winter). Some researchers have produced Fv antibodies (USP 4,642,334 to Moore, DNAX) and single chain Fv (SCFV) antibodies (see USP 4,946,778 to Ladner, Genex).

The above patent applications only show the production of antibody fragments in which some portion of the variable domains is coded for by nonhuman V gene regions. Humanized antibodies to date still retain various portions of light and heavy chain variable regions of nonhuman origin: the chimeric, Fv and single chain Fv antibodies retain the entire variable region of

nonhuman origin and CDR-grafted antibodies retain CDR of nonhuman origin.

Such nonhuman-derived regions are expected to elicit an immunogenic reaction when administered into a human patient (see Brüggemann *et al.* (1989), J. Exp. Med., 170:2153-2157; and Lo Buglio (1991), Sixth International Conference on Monoclonal Antibody Immunoconjugates for Cancer, San Diego, Ca). Thus, it is most desirable to obtain a human variable region which is capable of binding to a selected antigen.

One known human carcinoma tumor antigen is tumor-associated glycoprotein-72 (TAG-72), as defined by monoclonal antibody B72.3 (see Thor *et al.* (1986) Cancer Res., 46:3118-3124; and Johnson, *et al.* (1986), Cancer Res., 46:850-857). TAG-72 is associated with the surface of certain tumor cells of human origin, specifically the LS174T tumor cell line (American Type Culture Collection (ATCC) No. CL 188), which is a variant of the LS180 (ATCC No. CL 187) colon adenocarcinoma line.

Numerous murine monoclonal antibodies have been developed which have binding specificity for TAG-72. Exemplary murine monoclonal antibodies include the "CC" (colon cancer) monoclonal antibodies, which are a library of murine monoclonal antibodies developed using TAG-72 purified on an immunoaffinity column with an immobilized anti-TAG-72 antibody, B72.3 (ATCC HB-8108) (see EP 394277, to Schlom *et al.*, National Cancer Institute). Certain CC antibodies were deposited with the ATCC: CC49 (ATCC No. HB 9459); CC83 (ATCC No. HB 9453); CC46 (ATCC No. HB 9458); CC92 (ATCC No. HB 9454); CC30 (ATCC NO. HB 9457); CC11 (ATCC No. 9455) and CC15

(ATCC No. HB 9460). Various antibodies of the CC series have been chimerized (see, for example, EPO 0 365 997 to Mezes *et al.*, The Dow Chemical Company).

It is thus of great interest to develop antibodies against TAG-72 containing a light and/or heavy chain variable region(s) derived from human antibodies. However, the prior art simply does not teach recombinant and immunologic techniques capable of routinely producing an anti-TAG-72 antibody in which the light chain and/or the heavy chain variable regions have specificity and affinity for TAG-72 and which are derived from human sequences so as to elicit expectedly low or no HAMA response. It is known that the function of an immunoglobulin molecule is dependent on its three dimensional structure, which in turn is dependent on its primary amino acid sequence. A change of a few or even one amino acid can drastically affect the binding function of the antibody can drastically affect its the bidning affinity of the antibody, i.e., the resultant antibodies are generally presumed to be a non-specific immunoglobulin (NSI), i.e., lacking in antibody character, (see, for example, USP 4,816,567 to Cabilly *et al.*, Genentech).

Surprisingly, the present invention is capable of meeting many of these above mentioned needs and provides a method for supplying the desired antibodies. For example, in one aspect, the present invention provides a cell capable of expressing a composite antibody having binding specificity for TAG-72, said cell being transformed with (a) a DNA sequence encoding at least a portion of a light chain variable region (V_L) effectively homologous to the human Subgroup IV germline gene (Hum4 V_L); and a DNA sequence segment encoding at

least a portion of a heavy chain variable region (V_H) capable of combining with the V_L into a three dimensional structure having the ability to bind to TAG-72.

In another aspect, the present invention provides a composite antibody or antibody having binding specificity for TAG-72, comprising (a) a DNA sequence encoding at least a portion of a light chain (V_L) variable region effectively homologous to the human Subgroup IV germline gene (Hum4 V_L); and a DNA sequence segment encoding at least a portion of a heavy chain variable region (V_H) capable of combining with the V_L into a three dimensional structure having the ability to bind TAG-7.

The invention further includes the aforementioned antibody alone or conjugated to an imaging marker or therapeutic agent. The invention also includes a composition comprising the aforementioned antibody in unconjugated or conjugated form in a pharmaceutically acceptable, non-toxic, sterile carrier.

The invention is also directed to a method for *in vivo* diagnosis of cancer which comprises administering to an animal containing a tumor expressing TAG-72 a pharmaceutically effective amount of the aforementioned composition for the *in situ* detection of carcinoma lesions.

The invention is also directed to a method for intraoperative therapy which comprises (a) administering to patient containing a tumor expressing TAG-72 a pharmaceutically effective amount of the aforementioned

composition, whereby the tumor is localized, and (b) excising the localized tumors.

Additionally, the invention also concerns a process for preparing and expressing a composite antibody. Some of these processes are as follows. A process which comprises transforming a cell with a DNA sequence encoding at least a portion of a light chain variable region (V_L) effectively homologous to the human Subgroup IV germline gene (Hum4 V_L); and a DNA sequence segment encoding at least a portion of a heavy chain variable region (V_H) which is capable of combining with the V_L to form a three dimensional structure having the ability to bind to TAG-72. A process for preparing a composite antibody or antibody which comprises culturing a cell containing a DNA sequence encoding at least a portion of a light chain variable region (V_L) effectively homologous to the human Subgroup IV germline gene (Hum4 V_L); and a DNA sequence segment encoding at least a portion of a heavy chain variable region (V_H) capable of combining with the V_L into a three dimensional structure having the ability to bind to TAG-72 under sufficient conditions for the cell to express the immunoglobulin light chain and immunoglobulin heavy chain. A process for preparing an antibody conjugate comprising contacting the aforementioned antibody or antibody with an imaging marker or therapeutic agent.

Description of the Drawings

Figure 1 illustrates a basic immunoglobulin structure.

Figure 2 illustrates the nucleotide sequences of $V_H\alpha TAG$, CC46 V_H, CC49 V_H, CC83 V_H and CC92 V_H.

Figure 3 illustrates the amino acid sequences of $V_H\alpha TAG$, CC46 V_H, CC49 V_H, CC83 V_H and CC92 V_H.

Figure 4 illustrates the V_H nucleotide and amino acid sequences of antibody B17X2.

Figure 5 illustrates the mouse germline J-H genes from pNP9.

Figure 6 illustrates the plasmid map of p49g1-2.3.

Figure 7 illustrates the plasmid map of p83g1-2.3.

Figure 8 illustrates the entire sequence of HUMVL(+) and HUMVL(-).

Figure 9 illustrates the human J4 (HJ4) nucleotide sequence and amino acid sequence.

Figure 10 illustrates the nucleotide sequences, and the amino acid sequences of Hum4 V_L, *ClaI-Hin*dIII segment.

Figure 11 illustrates a schematic representation of the human germline Subgroup IV V_L gene (Hum4 V_L), as the target for the PCR.

Figure 12 shows the results of agarose gel electrophoresis of the PCR reaction to obtain the Hum4 V_L gene.

Figure 13 illustrates the restriction enzyme map of pRL1000, and precursor plasmids pSV2neo,

pSV2neo-101 and pSV2neo-102. "X" indicates where the *Hin*dIII site of pSV2neo has been destroyed.

Figure 14 illustrates a polylinker segment made by synthesizing two oligonucleotides: CH(+) and CH(-).

Figure 15 illustrates a primer, NEO102SEQ, used for sequencing plasmid DNA from several clones of pSV2neo-102.

Figure 16 illustrates an autoradiogram depicting the DNA sequence of the polylinker region in pSV2neo-102.

Figure 17 illustrates a partial nucleotide sequence segment of pRL1000.

Figure 18 illustrates the restriction enzyme map of pRL1001.

Figure 19 illustrates an autoradiogram of DNA sequence for pRL1001 clones.

Figure 20 illustrates a competition assay for binding to TAG-using a composite Hum4 V_L, $V_H\alpha$TAG antibody.

Figure 21 illustrates a general DNA construction of a single chain, composite Hum4 V_L, $V_H\alpha$TAG.

Figure 22 illustrates the nucleotide sequence and amino acid sequence of SCFV1.

Figure 23 shows the construction of plasmid pCGS515/SCFV1.

Figure 24 shows the construction of plasmid pSCFV31.

Figure 25 shows the construction of E. coli SCFV expression plasmids containing Hum4 V_L.

Figure 26 shows the DNA sequence and amino acid sequence of Hum4 V_L-CC49V_H SCFV present in pSCFVUHH.

Figure 27 shows the construction plasmid pSCFV UHH and a schematic of a combinatorial library of V_H genes with Hum4 V_L.

Figure 28 illustrates the nucleotide sequence of FLAG peptide adapter in pATDFLAG.

Figure 29 illustrates the construction of pATDFLAG, pHumVL-HumVH (X) and pSC49FLAG.

Figure 30 illustrates the nucleotide and amino acid sequences of pSC49FLAG.

Detailed Description of the Invention

Nucleic acids, amino acids, peptides, protective groups, active groups and so on, when abbreviated, are abbreviated according to the IUPAC IUB (Commission on Biological Nomenclature) or the practice in the fields concerned.

The basic immunoglobulin structural unit is set forth in Figure 1. The terms "constant" and "variable" are used functionally. The variable regions of both light (V_L) and heavy (V_H) chains determine binding recognition and specificity to the antigen. The constant region domains of light (C_L) and heavy (C_H) chains confer important biological properties such as

antibody chain association, secretion, transplacental mobility, complement binding, binding to Fc receptors and the like.

The immunoglobulins of this invention have been developed to address the problems of the prior art. The methods of this invention produce, and the invention is directed to, composite antibodies. By "composite antibodies" is meant immunoglobulins comprising variable regions not hitherto found associated with each other in nature. By, "composite Hum4 V_L, V_H antibody" means an antibody or immunoreactive fragment thereof which is characterized by having at least a portion of the V_L region encoded by DNA derived from the Hum4 V_L germline gene and at least a portion of a V_H region capable of combining with the V_L to form a three dimensional structure having the ability to bind to TAG-72.

The composite Hum4 V_L, V_H antibodies of the present invention assume a conformation having an antigen binding site which binds specifically and with sufficient strength to TAG-72 to form a complex capable of being isolated by using standard assay techniques (e.g., enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), or flourescence-activated cell sorter analysis (FACS), immunohistochemistry and the like). Preferably, the composite Hum4 V_L, V_H antibodies of the present invention have an antigen binding affinity or avidity greater than 10^5 M^{-1}, more preferably greater than 10^6 M^{-1} and most preferably greater than 10^8 M^{-1}. For a discussion of the techniques for generating and reviewing immunoglobulin binding affinities see Munson (1983), Methods Enzymol.,

92:543-577 and Scatchard (1949), Ann. N.Y. Acad. Sci., 51:660-672.

Human antibody kappa chains have been classified into four subgroups on the basis of invariant amino acid sequences (see, for example, Kabat *et al.* (1991), Sequences of Proteins of Immunological Interest (4th ed.), published by The U.S. Department of Health and Human Services). There appear to be approximately 80 human V_K genes, but only one Subgroup IV V_K gene has been identified in the human genome (see Klobeck, *et al.* (1985), Nucleic Acids Research, 13:6516-6528). The nucleotide sequence of Hum4 V_L is set forth in Kabat *et al.* (1991), *supra*; and Wang *et al.* (1973), Nature, 243:126-127.

It has been found, quite surprisingly, that an immunoglobulin having a light chain with at least a portion of the V_L encoded by a gene derived from Hum4 V_L may, if combined with a suitable V_H, have binding specificity for TAG-72.

The type of J_L gene segment selected is not critical to the invention, in that it is expected that any J_L, if present, can associate with the Hum4 V_L. The present invention obviously contemplates the Hum4 V_L in association with a human J_K sequence. The five human J_K sequences are set forth in Heiter *et al.* (1982), The Journal of Biological Chemistry, 357:1516-1522. However, the present invention is not intended to be limited to the human J_K. The present invention specifically contemplates the Hum4 V_L in association with any of the at least six human J_λ genes (see Hollis *et al.* (1982), Nature, 296:321-325).

An exemplary technique for engineering the Hum4 V_L with selected J_L segments includes synthesizing a primer having a so-called "wagging tail", that does not hybridize with the target DNA; thereafter, the sequences are amplified and spliced together by overlap extension (see Horton *et al.* (1989), Gene, 77:61-68).

The C_L of the composite Hum4 V_L, V_H antibodies is not critical to the invention. To date, the Hum4 V_L has only been reported as having been naturally rearranged with the single C_K gene (see Heiter *et al.* (1980), Cell, 22:197-207). However, the present invention is not intended to be limited to the C_K light chain constant domain. That is, the C_L gene segment may also be any of the at least six C_λ genes (see Hollis *et al.*, *supra*).

The DNA encoding the heavy chain variable region consists roughly of a heavy chain variable (V_H) gene sequence, a heavy chain diversity (D_H) gene sequence, and a heavy chain joining (J_H) gene sequence.

The present invention is directed to any V_H capable of combining with a light chain variable region effectively homologous to the light chain variable region encoded by the human Subgroup IV germline gene, to form a three dimensional structure having the ability to bind to TAG-72.

The choice of heavy chain diversity (D_H) segment and the heavy chain joining (J_H) segment of the composite Hum4 V_L, V_H antibody are not critical to the present invention. Obviously, human and murine D_H and J_H gene segments are contemplated, provided that a given combination does not significantly decrease binding to

TAG-72. Specifically, when utilizing CC46 V_H, CC49 V_H, CC83 V_H and CC92 V_H, the composite Hum4 V_L, V_H antibody will be designed to utilize the D_H and J_H segments which naturally associated with those V_H of the respective hybridomas (see Figures 2 and 3). Exemplary murine and human D_H and J_H sequences are set forth in Kabat *et al.* (1991), *supra*. An exemplary technique for engineering such selected D_H and J_H segments with a V_H sequence of choice includes synthesizing selected oligonucleotides, annealing and ligating in a cloning procedure (see, Horton *et al.*, *supra*).

In a specific embodiment the composite Hum4 V_L, V_H antibody will be a "composite Hum4 V_L, $V_H\alpha$TAG antibody", means an antibody or immunoreactive fragment thereof which is characterized by having at least a portion of the V_L region encoded by DNA derived from the Hum4 V_L germline gene and at least a portion of the V_H region encoded by DNA derived from the $V_H\alpha$TAG germline gene, which is known in the art (see, for example, EPO 0 365 997 to Mezes *et al.*, the Dow Chemical Company). Figure 2 shows the nucleotide sequence of $V_H\alpha$TAG, and the nucleotide sequences encoding the V_H of the CC46, CC49, CC83 and CC92 antibodies, respectively. Figure 3 shows the corresponding amino acid sequences of $V_H\alpha$TAG, CC46 V_H, CC49 V_H, CC83 V_H and CC92 V_H.

A comparison of the nucleotide and amino acid sequences of $V_H\alpha$TAG, CC46 V_H, CC49 V_H, CC83 V_H and CC92 V_H shows that those CC antibodies are derived from $V_H\alpha$TAG. Somatic mutations occurring during productive rearrangement of the V_H derived from $V_H\alpha$TAG in a B cell gave rise to some nucleotide changes that may or may not

result in a homologous amino acid change between the productively rearranged hybridomas (see, EPO 0 365 997).

Because the nucleotide sequences of $V_H\alpha TAG$ and Hum4 V_L germline genes have been provided herein, the present invention is intended to include other antibody genes which are productively rearranged from the $V_H\alpha TAG$ germline gene. Other antibodies encoded by DNA derived from $V_H\alpha TAG$ may be identified by using a hybridization probe made from the DNA or RNA of the $V_H\alpha TAG$ or rearranged genes containing the recombined $V_H\alpha TAG$. Specifically, the probe will include of all or a part of the $V_H\alpha TAG$ germline gene and its flanking regions. By "flanking regions" is meant to include those DNA sequences from the 5' end of the $V_H\alpha TAG$ to the 3' end of the upstream gene, and from 3' end of the $V_H\alpha TAG$ to the 5' end of the downstream gene.

The CDR from the variable region of antibodies derived from $V_H\alpha TAG$ may be grafted onto the FR of selected V_H, i.e., FR of a human antibody (see EPO 0 239 400 to Winter). For example, the cell line, B17X2, expresses an antibody utilizing a variable light chain encoded by a gene derived from Hum4 V_L and a variable heavy chain which makes a stable V_L and V_H combination (see Marsh *et al.* (1985), Nucleic Acids Research, 13:6531-6544; and Polke *et al.* (1982), Immunobiol. 163:95-109. The nucleotide sequence of the V_H chain for B17X2 is shown in Figure 4. The B17X2 cell line is publicly available from Dr. Christine Polke, Universitäts-Kinderklinik, Josef-Schneider-Str. 2, 8700 Würzburg, FRG). B17X2 is directed to N-Acetyl-D-Glucosamine and is not specific for TAG-72.

However, consensus sequences of antibody derived from the CDR1 of $V_H\alpha$TAG (amino acid residues 31 to 35 of Figure 3) may be inserted into B17X2 (amino acid residues 31 to 37 of Figure 4) and the CDR2 of $V_H\alpha$TAG (amino residues 50 to 65 of Figure 3) may be inserted into B17X2 (amino acid residues 52 to 67 of Figure 4). The CDR3 may be replaced by any D_H and J_H sequence which does not affect the binding of the antibody for TAG-72 but, specifically, may be replaced by the CDR3 of an antibody having its V_H derived from $V_H\alpha$TAG, e.g., CC46, CC49, CC83 and CC92. Exemplary techniques for such replacement are set forth in Horton *et al.*, *supra*.

The C_H domains of immunoglobulin heavy chain derived from $V_H\alpha$TAG genes, for example may be changed to a human sequence by known techniques (see, USP 4,816,567 to Cabilly, Genentech). C_H domains may be of various complete or shortened human isotypes, i.e., IgG (e.g., IgG_1, IgG_2, IgG_3, and IgG_4), IgA (e.g., IgA1 and IgA2), IgD, IgE, IgM, as well as the various allotypes of the individual groups (see Kabat *et al.* (1991), *supra*).

Given the teachings of the present invention, it should be apparent to the skilled artisan that human V_H genes can be tested for their ability to produce an anti-TAG-72 immunoglobilin combination with the Hum4 V_L gene. The V_L may be used to isolate a gene encoding for a V_H having the ability to bind to TAG-72 to test myriad combinations of Hum4 V_L and V_H that may not naturally occur in nature, e.g., by generating a combinatorial library using the Hum4 V_L gene to select a suitable V_H. Examples of these enabling technologies include screening of combinatorial libraries of V_L-V_H combinations using an Fab or single chain antibody

(SCFV) format expressed on the surfaces of fd phage (Clackson, *et al.* (1991), Nature, 352:624-628), or using a λ phage system for expression of Fv's or Fabs (Huse, *et al.* (1989), Science, 246:1275-1281). However, according to the teachings set forth herein, it is now possible to clone SCFV antibodies in *E.coli*, and express the SCFVs as secreted soluble proteins. SCFV proteins produced in *E. coli* that contain a Hum4 V_L gene can be screened for binding to TAG-72 using, for example, a two-membrane filter screening system (Skerra, *et al.* (1991), Analytical Biochemistry, 196:151-155).

The desired gene repertoire can be isolated from human genetic material obtained from any suitable source, e.g., peripheral blood lymphocytes, spleen cells and lymph nodes of a patient with tumor expressing TAG-72. In some cases, it is desirable to bias the repertoire for a preselected activity, such as by using as a source of nucleic acid, cells (source cells) from vertebrates in any one of various stages of age, health and immune response.

Cells coding for the desired sequence may be isolated, and genomic DNA fragmented by one or more restriction enzymes. Tissue (e.g., primary and secondary lymph organs, neoplastic tissue, white blood cells from peripheral blood and hybridomas) from an animal exposed to TAG-72 may be probed for selected antibody producing B cells. Variability among B cells derived from a common germline gene may result from somatic mutations occurring during productive rearrangement.

Generally, a probe made from the genomic DNA of a germline gene or rearranged gene can be used by those

skilled in the art to find homologous sequences from unknown cells. For example, sequence information obtained from Hum4 V_L and $V_H\alpha$TAG may be used to generate hybridization probes for naturally-occurring rearranged V regions, including the 5' and 3' nontranslated flanking regions. The genomic DNA may include naturally-occurring introns for portions thereof, provided that functional splice donor and splice acceptor regions had been present in the case of mammalian cell sources.

Additionally, the DNA may also be obtained from a cDNA library. mRNA coding for heavy or light chain variable domain may be isolated from a suitable source, either mature B cells or a hybridoma culture, employing standard techniques of RNA isolation. The DNA or amino acids also may be synthetically synthesized and constructed by standard techniques of annealing and ligating fragments (see Jones, *et al.* (1986), Nature, 321:522-525; Reichmann *et al.*, (1988), Nature, 332:323-327; Sambrook *et al.* (1989), *supra* and Merrifield *et al.* (1963), J. Amer. Chem. Soc., 85:2149-2154). Heavy and light chains may be combined *in vitro* to gain antibody activity (see Edelman, *et al.* (1963), Proc. Natl. Acad. Sci. USA, 50:753).

The present invention also contemplates a gene library of $V_H\alpha$TAG homologs, preferably human homologs of $V_H\alpha$TAG. By "homolog" is meant a gene coding for a V_H region (not necessarily derived from, or even effectively homologous to, the $V_H\alpha$TAG germline gene) capable of combining with a light chain variable region effectively homologous to the light chain variable region encoded by the human Subgroup IV germline gene.

to form a three dimensional structure having the ability to bind to TAG-72.

Preferably, the gene library is produced by a primer extension reaction or combination of primer extension reactions as described herein. The $V_H\alpha$TAG homologs are preferably in an isolated form, that is, substantially free of materials such as, for example, primer extension reaction agents and/or substrates, genomic DNA segments, and the like. The present invention thus is directed to cloning the $V_H\alpha$TAG-coding DNA homologs from a repertoire comprised of polynucleotide coding strands, such as genomic material containing the gene expressing the variable region or the messenger RNA (mRNA) which represents a transcript of the variable region. Nucleic acids coding for $V_H\alpha$TAG-coding homologs can be derived from cells producing IgA, IgD, IgE, IgG or IgM, most preferably from IgM and IgG, producing cells.

The $V_H\alpha$TAG-coding DNA homologs may be produced by primer extension. The term "primer" as used herein refers to a polynucleotide whether purified from a nucleic acid restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complimentary to a nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase, reverse transcriptase and the like, and at a suitable temperature and pH.

Preferably, the $V_H\alpha$TAG-coding DNA homologs may be produced by polymerase chain reaction (PCR) amplification of double stranded genomic or cDNA, wherein two

primers are used for each coding strand of nucleic acid to be exponentially amplified. The first primer becomes part of the nonsense (minus or complementary) strand and hybridizes to a nucleotide sequence conserved among V_H (plus) strands within the repertoire. PCR is described in Mullis *et al.* (1987), Meth. Enz., 155:335-350; and PCR Technology, Erlich (ed.) (1989). PCR amplification of the mRNA from antibody-producing cells is set forth in Orlandi *et al.* (1989), Proc. Natl. Acad. Sci.. USA, 86:3387-3837.

According to a preferred method, the $V_H\alpha$TAG-coding DNA homologs are connected via linker to form a SCFV having a three dimensional structure capable of binding TAG-72. The SCFV construct can be in a V_L-L-V_H or V_H-L-V_L configuration. For a discussion of SCFV see Bird *et al.* (1988), Science, 242:423-426. The design of suitable peptide linker regions is described in U.S. Patent No. 4,704,692 to Ladner *et al.*. Genex.

The nucleotide sequence of a primer is selected to hybridize with a plurality of immunoglobulin heavy chain genes at a site substantially adjacent to the $V_H\alpha$TAG-coding DNA homolog so that a nucleotide sequence coding for a functional (capable of binding) polypeptide is obtained. The choice of a primer's nucleotide sequence depends on factors such as the distance on the nucleic acid from the region coding for the desired receptor, its hybridization site on the nucleic acid relative to any second primer to be used, the number of genes in the repertoire it is to hybridize to, and the like. To hybridize to a plurality of different nucleic acid strands of $V_H\alpha$TAG-coding DNA homolog, the primer

must be a substantial complement of a nucleotide sequence conserved among the different strands.

The peptide linker may be coded for by the nucleic acid sequences that are part of the polynucleotide primers used to prepare the various gene libraries. The nucleic acid sequence coding for the peptide linker can be made up of nucleic acids attached to one of the primers or the nucleic acid sequence coding for the peptide linker may be derived from nucleic acid sequences that are attached to several polynucleotide primers used to create the gene libraries. Additionally, noncomplementary bases or longer sequences can be interspersed into the primer, provided the primer sequence has sufficient complementarily with the sequence of the strand to be synthesized or amplified to non-randomly hybridize therewith and thereby form an extension product under polynucleotide synthesizing conditions (see Horton *et al.* (1989), Gene, 77:61-68).

Exemplary human V_H sequences from which complementary primers may be synthesized are set forth in Kabat *et al.* (1991), *supra*: Humphries *et al.* (1988), Nature, 331:446-449; Schroeder *et al.* (1990), Proc. Natl. Acad. Sci. USA, 87:6146-6150; Berman *et al.* (1988), EMBO Journal, 7:727-738; Lee *et al.* (1987), J. Mol. Biol., 195:761-768); Marks *et al.* (1991), Eur. J. Immunol., 21:985-991; Willems, *et al.* (1991), J. Immunol., 146:3646-3651; and Person *et al.* (1991), Proc Natl. Acad. Sci. USA, 88:2432-2436. To produce V_H coding DNA homologs, first primers are therefore chosen to hybridize to (i.e. be complementary to) conserved regions within the J region, CH1 region, hinge region, CH2 region, or CH3 region of immunoglobulin genes and the like. Second primers are

therefore chosen to hydribidize with a conserved nucleotide sequence at the 5' end of the $V_H\alpha$TAG-coding DNA homolog such as in that area coding for the leader or first framework region.

Alternatively, the nucleic acid sequences coding for the peptide linker may be designed as part of a suitable vector. As used herein, the term "expression vector" refers to a nucleic acid molecule capable of directing the expression of genes to which they are operatively linked. The choice of vector to which a $V_H\alpha$TAG-coding DNA homologs is operatively linked depends directly, as is well known in the art, on the functional properties desired, e.g., replication or protein expression, and the host cell (either procaryotic or eucaryotic) to be transformed, these being limitations inherent in the art of constructing recombinant DNA molecules. In preferred embodiments, the eucaryotic cell expression vectors used include a selection marker that is effective in an eucaryotic cell, preferably a drug resistant selection marker.

Expression vectors compatible with procaryotic cells are well known in the art and are available from several commercial sources. Typical of vector plasmids suitable for procaryotic cells are pUC8, pUC9, pBR322, and pBR329 available from BioRad Laboratories, (Richmond, CA), and pPL and pKK223 available from Pharmacia, (Piscataway, NJ).

Expression vectors compatible with eucaryotic cells, preferably those compatible with vertebrate cells, can also be used. Eucaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are

provided containing convenient restriction sites for insertion of the desired DNA homologue. Typical of vector plasmids suitable for eucaryotic cells are pSV2neo and pSV2gpt (ATCC), pSVL and pKSV-10 (Pharmacia), pBPV-1/PML2d (International Biotechnologies, Inc.), and pTDT1 (ATCC).

The use of viral expression vectors to express the genes of the $V_{H\alpha}$TAG-coding DNA homologs is also contemplated. As used herein, the term "viral expression vector" refers to a DNA molecule that includes a promoter sequences derived from the long terminal repeat (LTR) region of a viral genome. Exemplary phage include λ phage and fd phage (see, Sambrook, *et al.* (1989), Molecular Cloning: A Laboratory Manual. (2nd ed.), and McCafferty *et al.* (1990), Nature, 6301:552-554.

The population of $V_{H\alpha}$TAG-coding DNA homologs and vectors are then cleaved with an endonuclease at shared restriction sites. A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini. For instance, complementary cohesive termini can be engineered into the $V_{H\alpha}$TAG-coding DNA homologs during the primer extension reaction by use of an appropriately designed polynucleotide synthesis primer, as previously discussed. The complementary cohesive termini of the vector and the DNA homolog are then operatively linked (ligated) to produce a unitary double stranded DNA molecule.

The restriction fragments of Hum4 V_L-coding DNA and the $V_{H\alpha}$TAG-coding DNA homologs population are randomly ligated to the cleaved vector. A diverse,

random population is produced with each vector having a $V_H\alpha$TAG-coding DNA homolog and Hum4 V_L-coding DNA located in the same reading frame and under the control of the vector's promoter.

The resulting single chain construct is then introduced into an appropriate host to provide amplification and/or expression of a composite Hum4 V_L, $V_H\alpha$TAG homolog single chain antibody. Transformation of appropriate cell hosts with a recombinant DNA molecule of the present invention is accomplished by methods that typically depend on the type of vector used. With regard to transformation of procaryotic host cells, see, for example, Cohen *et al.* (1972), Proceedings National Academy of Science, USA, 69:2110; and Sambrook, *et al.* (1989), *supra*. With regard to the transformation of vertebrate cells with retroviral vectors containing rDNAs, see for example, Sorge *et al.* (1984), Mol. Cell. Biol., 4:1730-1737; Graham *et al.* (1973), Virol., 52:456; and Wigler *et al.* (1979), Proceedings National Academy of Sciences, USA, 76:1373-1376.

Exemplary prokaryotic strains that may be used as hosts include *E. coli*, *Bacilli*, and other enterobacteriaceae such as *Salmonella typhimurium*, and various *Pseudomonas*. Common eukaryotic microbes include *S. cerevisiae* and *Pichia pastoris*. Common higher eukaryotic host cells include Sp2/0, VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 and MDCK cell lines. Furthermore, it is now also evident that any cell line producing Hum4 V_L, e.g., the B17X2 human cell line, can be used as a recipient human cell line for introduction of a V_H gene complementary to the Hum4 V_L which allows binding to TAG-72. For example, the B17X2 heavy chain may be genetically modified to not

produce the endogenous heavy chain by well known methods; in this way, glycosylation patterns of the antibody produced would be human and not non-human derived.

Successfully transformed cells, i.e., cells containing a gene encoding a composite Hum4 V_L, $V_H\alpha$TAG homolog single chain antibody operatively linked to a vector, can be identified by any suitable well known technique for detecting the binding of a receptor to a ligand. Preferred screening assays are those where the binding of the composite Hum4 V_L, $V_H\alpha$TAG homolog single chain antibody to TAG-72 produces a detectable signal, either directly or indirectly. Screening for productive Hum4 V_L and $V_H\alpha$TAG homolog combinations, or in other words, testing for effective antigen binding sites to TAG-72 is possible by using for example, a radiolabeled or biotinylated screening agent, e.g., antigens, antibodies (e.g., B72.3, CC49, CC83, CC46, CC92, CC30, CC11 and CC15) or anti-idiotypic antibodies (see Huse *et al.*, *supra*, and Sambrook *et al.*, *supra*); or the use of marker peptides to the NH_2- or COOH-terminus of the SCFV construct (see Hopp *et al.* (1988). Biotechnology, 6:1204-1210).

Of course, the Hum4 V_L-coding DNA and the $V_H\alpha$TAG-coding DNA homologs may be expressed as individual polypeptide chains (e.g.. Fv) or with whole or fragmented constant regions (e.g., Fab, and $F(ab')_2$). Accordingly. the Hum4 V_L-coding DNA and the $V_H\alpha$TAG-coding DNA homologs may be individually inserted into a vector containing a C_L or C_H or fragment thereof, respectively. For a teaching of how to prepare suitable

vectors see EPO 0 365 997 to Mezes *et al.*, The Dow Chemical Company.

DNA sequences encoding the light chain and heavy chain of the composite Hum4 V_L, V_H antibody may be inserted into separate expression vehicles, or into the same expression vehicle. When coexpressed within the same organism, either on the same or the different vectors, a functionally active Fv is produced. When the V_HαTAG-coding DNA homolog and Hum4 V_L polypeptides are expressed in different organisms, the respective polypeptides are isolated and then combined in an appropriate medium to form a Fv. See Greene *et al.*, Methods in Molecular Biology, Vol. 9, Wickner *et al.* (ed.); and Sambrook *et al.*, *supra*).

Subsequent recombinations can be effected through cleavage and removal of the Hum4 V_L-coding DNA sequence to use the V_HαTAG-coding DNA homologs to produce Hum4 V_L-coding DNA homologs. To produce a Hum4 V_L-coding DNA homolog, first primers are chosen to hybridize with (i.e. be complementary to) a conserved region within the J region or constant region of immunoglobulin light chain genes and the like. Second primers become part of the coding (plus) strand and hybridize to a nucleotide sequence conserved among minus strands. Hum4 V_L-coding DNA homologs are ligated into the vector containing the V_HαTAG-coding DNA homolog, thereby creating a second population of expression vectors. The present invention thus is directed to cloning the Hum4 V_L-coding DNA homologs from a repertoire comprised of polynucleotide coding strands, such as genomic material containing the gene expressing the variable region or the messenger RNA (mRNA) which represents a transcript of the variable region. It is

thus possible to use an iterative process to define yet further, composite antibodies, using later generation $V_H\alpha$TAG-coding DNA homologs and Hum4 V_L-coding DNA homologs.

The present invention further contemplates genetically modifying the antibody variable and constant regions to include effectively homologous variable region and constant region amino acid sequences. Generally, changes in the variable region will be made in order to improve or otherwise modify antigen binding properties of the receptor. Changes in the constant region of the antigen receptor will, in general, be made in order to improve or otherwise modify biological properties, such as complement fixation, interaction with membranes, and other effector functions.

"Effectively homologous" refers to the concept that differences in the primary structure of the variable region may not alter the binding characteristics of the antigen receptor. Normally, a DNA sequence is effectively homologous to a second DNA sequence if at least 70 percent, preferably at least 80 percent, and most preferably at least 90 percent of the active portions of the DNA sequence are homologous. Such changes are permissable in effectively homologous amino acid sequences so long as the resultant antigen receptor retains its desired property.

If there is only a conservative difference between homologous positions of sequences, they may be regarded as equivalents under certain circumstances. General categories of potentially equivalent amino acids are set forth below, wherein, amino acids within a group may be substituted for other amino acids in that group:

(1) glutamic acid and aspartic acid; (2) hydrophobic amino acids such as alanine, valine, leucine and isoleucine; (3) asparagine and glutamine; (4) lysine, arginine; and (5) threonine and serine.

Exemplary techniques for nucleotide replacement include the addition, deletion, or substitution of various nucleotides, deletion or substitution of various nucleotides, provided that the proper reading frame is maintained. Exemplary techniques include using polynucleotide-mediated, site-directed mutagenesis, i.e., using a single strand as a template for extension of the oligonucleotide to produce a strand containing the mutation (see Zoller *et al.* (1982), Nuc. Acids Res., 10:6487-6500; Norris et al. (1983), Nuc. Acids Res., 11:5103-5112; Zoller et al. (1984), DNA, 3:479-488; Kramer *et al.* (1982), Nuc. Acids Res., 10:6475-6485 and polymerase chain reaction, i.e., exponentially amplifying DNA *in vitro* using sequence specified oligonucleotides to incorporate selected changes (see PCR Technology: Principles and Applications for DNA Amplification, Erlich, (ed.) (1989); and Horton *et al. supra*).

Further, the antibodies may have their constant region domain modified, ie., the C_L, CH_1, hinge, CH_2, CH_3 and/or CH_4 domains of an antibody polypeptide chain may be deleted, inserted or changed (see EPO 327 378 A1 to Morrison *et al.*, the Trustees of Columbia University; USP 4,642,334 to Moore *et al.*, DNAX: and USP 4,704,692 to Ladner *et al.*, Genex).

Once a final DNA construct is obtained, the composite Hum4 V_L, V_H antibodies may be produced in large quantities by injecting the host cell into the

peritoneal cavity of pristane-primed mice, and after an appropriate time (about 1-2 weeks), harvesting ascites fluid from the mice, which yields a very high titer of homogeneous composite Hum4 V_L, V_H antibodies, and isolating the composite Hum4 V_L, V_H antibodies by methods well known in the art (see Stramignoni. *et al.* (1983), Intl. J. Cancer, 31:543-552). The host cell are grown *in vivo*, as tumors in animals, the serum or ascites fluid of which can provide up to about 50 mg/mL of composite Hum4 V_L, V_H antibodies. Usually, injection (preferably intraperitoneal) of about 10^6 to 10^7 histocompatible host cells into mice or rats will result in tumor formation after a few weeks. It is possible to obtain the composite Hum4 V_L, V_H antibodies from a fermentation culture broth of procaryotic and eucaryotic cells, or from inclusion bodies of *E.coli* cells (see Buckholz and Gleeson (1991), BIO/TECHNOLOGY, 9:1067-1072. The composite Hum4 V_L, V_H antibodies can then be collected and processed by well-known methods (see generally, Immunological Methods, vols. I & II, eds. Lefkovits, I. and Pernis, B., (1979 & 1981) Academic Press, New York, N.Y.: and Handbook of Experimental Immunology, ed. Weir, D., (1978) Blackwell Scientific Publications, St. Louis, MO.)

The composite Hum4 V_L, V_H antibodies can then be stored in various buffer solutions such as phosphate buffered saline (PBS), which gives a generally stable antibody solution for further use.

Uses

The composite Hum4 V_L, V_H antibodies provide unique benefits for use in a variety of cancer treatments. In addition to the ability to bind

specifically to malignant cells and to localize tumors and not bind to normal cells such as fibroblasts, endothelial cells, or epithelial cells in the major organs, the composite Hum4 V_L, V_H antibodies may be used to greatly minimize or eliminate ANHA responses thereto. Moreover, TAG-72 contains a variety of epitopes and thus it may be desirable to administer several different composite Hum4 V_L, V_H antibodies which utilize a variety of V_H in combination with Hum4 V_L.

Specifically, the composite Hum4 V_L, V_H antibodies are useful for, but not limited to, *in vivo* and *in vitro* uses in diagnostics, therapy, imaging and biosensors.

The composite Hum4 V_L, V_H antibodies may be incorporated into a pharmaceutically acceptable, non-toxic, sterile carrier. Injectable compositions of the present invention may be either in suspension or solution form. In solution form the complex (or when desired the separate components) is dissolved in a pharmaceutically acceptable carrier. Such carriers comprise a suitable solvent, preservatives such as benzyl alcohol, if needed, and buffers. Useful solvents include, for example, water, aqueous alcohols, glycols, and phosphonate or carbonate esters. Such aqueous solutions generally contain no more than 50 percent of the organic solvent by volume.

Injectable suspensions require a liquid suspending medium, with or without adjuvants, as a carrier. The suspending medium can be, for example, aqueous polyvinyl-pyrrolidone, inert oils such as vegetable oils or highly refined mineral oils, or aqueous carboxymethlycellulose. Suitable physio-

logically-acceptable adjuvants, if necessary to keep the complex in suspension, may be chosen from among thickeners such as carboxymethylcellulose, polyvinyl-pyrrolidone, gelatin, and the alginates. Many surfactants are also useful as suspending agents, for example, lecithin, alkylphenol, polyethylene oxide adducts, naphthalenesulfonates, alkylbenzenesulfonates, and the polyoxyethylene sorbitan esters. Many substances which effect the hydrophibicity, density, and surface tension of the liquid suspension medium can assist in making injectable suspensions in individual cases. For example, silicone antifoams, sorbitol, and sugars are all useful suspending agents.

Methods of preparing and administering conjugates of the composite Hum4 V_L, V_H antibody, and a therapeutic agent are well known to or readily determined. Moreover, suitable dosages will depend on the age and weight of the patient and the therapeutic agent employed and are well known or readily determined.

Conjugates of a composite Hum4 V_L, V_H antibody and an imaging marker may be administered in a pharmaceutically effective amount for the *in vivo* diagnostic assays of human carcinomas, or metastases thereof, in a patient having a tumor that expresses TAG-72 and then detecting the presence of the imaging marker by appropriate detection means.

Administration and detection of the conjugates of the composite Hum4 V_L, V_H antibody and an imaging marker, as well as methods of conjugating the composite Hum4 V_L, V_H antibody to the imaging marker are accomplished by methods readily known or readily determined. The dosage of such conjugate will vary

depending upon the age and weight of the patient. Generally, the dosage should be effective to visualize or detect tumor sites, distinct from normal tissues. Preferably, a one-time dosage will be between 0.1 mg to 200 mg of the conjugate of the composite Hum4 V_L antibody and imaging marker per patient.

Examples of imaging markers which can be conjugated to the composite Hum4 V_L antibody are well known and include substances which can be detected by diagnostic imaging using a gamma scanner or hand held gamma probe, and substances which can be detected by nuclear magnetic resonance imaging using a nuclear magnetic resonance spectrometer.

Suitable, but not limiting, examples of substances which can be detected using a gamma scanner include ^{125}I, ^{131}I, ^{123}I, ^{111}In, ^{105}Rh, ^{153}Sm, ^{67}Cu, ^{67}Ga, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re and ^{99m}Tc. An example of a substance which can be detected using a nuclear magnetic resonance spectrometer is gadolinium.

Conjugates of a composite Hum4 V_L, V_H antibodies and a therapeutic agent may be administered in a pharmaceutically effective amount for the *in vivo* treatment of human carcinomas, or metastases thereof, in a patient having a tumor that expresses TAG-72. A "pharmaceutically effective amount" of the composite Hum4 V_L antibody means the amount of said antibody (whether unconjugated. i.e., a naked antibody, or conjugated to a therapeutic agent) in the pharmaceutical composition should be sufficient to achieve effective binding to TAG-72.

Exemplary naked antibody therapy includes, for example, administering heterobifunctional composite Hum4 V_L, V_H antibodies coupled or combined with another antibody so that the complex binds both to the carcinoma and effector cells, e.g., killer cells such as T cells, or monocytes. In this method, the composite Hum4 V_L antibody-therapeutic agent conjugate can be delivered to the carcinoma site thereby directly exposing the carcinoma tissue to the therapeutic agent. Alternatively, naked antibody therapy is possible in which antibody dependent cellular cytoxicity or complement dependent cytotoxicity is mediated by the composite Hum4 V_L antibody.

Examples of the antibody-therapeutic agent conjugates which can be used in therapy include antibodies coupled to radionuclides, such as ^{131}I, ^{90}Y, ^{105}Rh, ^{47}Sc, ^{67}Cu, ^{212}Bi, ^{211}At, ^{67}Ga, ^{125}I, ^{186}Re, ^{188}Re, ^{177}Lu, ^{99m}Tc, ^{153}Sm, ^{123}I and ^{111}In; to drugs, such as methotrexate, adriamycin; to biological response modifiers, such as interferon and to toxins, such as ricin.

Methods of preparing and administering conjugates of the composite Hum4 V_L, V_H antibodies and a therapeutic agent are well known or readily determined. The pharmaceutical composition may be administered in a single dosage or multiple dosage form. Moreover, suitable dosages will depend on the age and weight of the patient and the therapeutic agent employed and are well known or readily determined.

Composite Hum4 V_L, V_H antibodies, and particularly composite Hum4 V_L, V_H single chain antibodies thereof, are particularly suitable for radioimmunoguided

surgery (RIGS). In RIGS, an antibody labeled with an imaging marker is injected into a patient having a tumor that expresses TAG-72. The antibody localizes to the tumor and is detected by a hand-held gamma detecting probe (GDP). The tumor is then excised (see Martin *et al.* (1988), Amer. J. Surg., 156:386-392; and Martin *et al.* (1986), Hybridoma, 5:S97-S108). An exemplary GDP is the Neoprobe™ scanner, commercially available from Neoprobe Corporation, Columbus, OH. The relatively small size and human character of the composite Hum4 V_L, V_H single chain antibodies will accelerate whole body clearance and thus reduce the waiting period after injection before surgery can be effectively initiated.

Administration and detection of the composite Hum4 V_L, V_H antibody-imaging marker conjugate may be accomplished by methods well-known or readily determined.

The dosage will vary depending upon the age and weight of the patient, but generally a one time dosage of about 0.1 to 200 mg of antibody-marker conjugate per patient is administered.

EXAMPLES

The following nonlimiting examples are merely for illustration of the construction and expression of composite Hum4 V_L, V_H antibodies. All temperatures not otherwise indicated are Centigrade. All percents not otherwise indicated are by weight.

Example I

CC49 and CC83 were isolated from their respective hybridomas using pNP9 as a probe (see Figure 5). CC49 V_H was obtained from p49 g1-2.3 (see Figure 6) and CC83 V_H was obtained from p83 g1-2.3 (see Figure 7), following the procedures set forth in EPO 0 365 997.

DNA encoding an antibody light chain was isolated from a sample of blood from a human following the protocol of Madisen *et.al.* (1987), Am. J. Med. Genet., 27:379-390) with several modifications. Two 5 ml purple-cap Vacutainer tubes (containing EDTA as an anticoagulant) were filled with blood and stored at ambient temperature for 2 hours. The samples were transferred to two 4.5 mL centrifuge tubes. To each tube was added 22.5 mL of filter-sterilized erythrocycte lysate buffer (0.155 M NH_4Cl and 0.17 M Tris, pH 7.65, in a volume ratio of 9:1), and incubated at 37°C for 6.5 minutes. The tubes became dark red due to the lysed red blood cells. The samples were centrifuged at 9°C for 10 minutes, using an SS-34 rotor and a Sorvall centrifuge at 5,300 revolutions per minute (rpm) (~3,400 X g). The resulting white cell pellets were resuspended in 25 mL of 0.15 M NaCl solution. The white blood cells were then centrifuged as before. The pellets were resuspended in 500 µL of 0.15 M NaCl and transferred to

1.5 mL microcentrifuge tubes. The cells were pelleted again for 3 minutes, this time in the microcentrifuge at 3,000 rpm. Very few red blood cells remained on the pellet. After the supernatants were decanted from the two microcentrifuge tubes, 0.6 mL high TE buffer (100 mM Tris, pH 8.0) was added. The tubes were hand-shaken for 10 and 15 minutes. The resulting viscous solution was extracted with phenol, phenol-chloroform and finally with just chloroform as described in Sambrook *et al.*, *supra*. To 3.9 mL of pooled extracted DNA solution was added 0.4 mL NaOAc (3 M, pH 5), and 10 mL 100 percent ethanol. A white stringy precipitate was recovered with a yellow pipette tip, transferred into a new Eppendorf tube, washed once with 70 percent ethanol, and finally washed with 100 percent ethanol. The DNA was dried *in vacuo* for 1 minute and dissolved in 0.75 mL deionized water. A 20 µL aliquot was diluted to 1.0 mL and the OD 260 nm value was measured and recorded. The concentration of DNA in the original solution was calculated to be 0.30 mg/mL.

Oligonucleotides (oligos) were synthesized using phosphoramidite chemistry on a 380A DNA synthesizer (Applied Biosystems, Foster, CA) starting on 0.2 µM solid support columns. Protecting groups on the final products were removed by heating in concentrated ammonia solution at 55°C for 12 hours. Crude mixtures of oligonucleotides (approximately 12 OD 260 nm units) were applied to 16 percent polyacrylamide-urea gels and electrophoresed. DNA in the gels was visualized by short wave UV light. Bands were cut out and the DNA eluted by heating the gel pieces to 65°C for 2 hours. Final purification was achieved by application of the eluted DNA solution onto C-18 Sep-Pac™ columns

(Millipore) and elution of the bound oligonucleotide with a 60 percent methanol solution. The pure DNA was dissolved in deionized distilled water (ddH_2O) and quantitated by measuring OD 260 nm.

A GeneAmp™ DNA amplification kit (Cetus Corp., Emeryville, CA) was used to clone the Hum4 V_L germline gene by the PCR which was set up according to the manufacturer's directions. A thermal cycler was used for the denaturation (94 °C), annealing (45 °C) and elongation (72 °C) steps. Each of the three steps in a cycle were carried out for 4 minutes; there was a total of 30 cycles.

Upstream of the regulatory sequences in the Hum4 V_L germline gene, there is a unique *Cla* I restriction enzyme site. Therefore, the 5' end oligonucleotide for the PCR technique, called HUMVL(+) (Figure 8), was designed to include this *Cla* I site.

The 3' end oligonucleotide, called HUMVL(-) (Figure 8), contained a unique *Hind* III site; sufficient mouse intron sequence past the splicing site to permit an effective splice donor function; a human J4 sequence contiguous with the 3' end of the V_L exon of Hum4 V_L to complete the CDR3 and FR4 sequences of the V_L domain (see Figures 9 and 10); nucleotides to encode a tyrosine residue at position 94 in CDR3; and 29 nucleotides close to the 3' end of the V_L exon of Hum4 V_L (shown underlined in the oligonucleotide HUMVL(-) in Figure 8) to anneal with the human DNA target. In total, this 3' end oligonucleotide for the PCR was 98 bases long with a non-annealing segment (a "wagging tail") of 69 nucleotides. A schematic of the Hum4 V_L gene target and

the oligonucleotides used for the PCR are shown in Figure 11.

A PCR reaction was set up with 1 µg of total human DNA in a reaction volume of 100 µL. Primers HUMVL(-) and HUMVL(+) were each present at an initial concentratiuon of 100 pmol. Prior to the addition of *Taq* polymerase (2.5 units/reaction) 100 µLs of mineral oil were used to overlay the samples. Control samples were set up as outlined below. The samples were heated to 95 °C for 3 minutes. When the PCR was complete, 20 µL samples were removed for analysis by agarose gel electrophoresis.

Based on the known size of the Hum4 V_L DNA fragment to be cloned, and the size of the oligonucleotides used to target the gene, a product of 1099 bp was expected. A band corresponding to this size was obtained in the reaction (shown in lane 7, Figure 12).

To prepare a plasmid suitable for cloning and subsequently expressing the Hum4 V_L gene, the plasmid pSV2neo was obtained from ATCC and subsequently modified. pSV2neo was modified as set forth below (see Figure 13).

The preparation of pSV2neo-101 was as follows. Ten micrograms of purified pSV2neo were digested with 40 units of *Hind* III at 37 °C for 1 hour. The linearized plasmid DNA was precipitated with ethanol, washed, dried and dissolved in 10 µL water. Two microliters each of 10 mM dATP, dCTP, dGTP and dTTP were added, as well as 2 µL of 10X ligase buffer. Five units (1 µL) of DNA polymerase I were added to make blunt the Hind III

sticky ends. The reaction mixture was incubated at room temperature for 30 minutes. The enzyme was inactivated by heating the mixture to 65°C for 15 minutes. The reaction mixture was phenol extracted and ethanol precipitated into a pellet. The pellet was dissolved in 20 µl deionized, distilled water. A 2 µl aliquot (ca. 1 µg) was then added to a standard 20 µL ligation reaction, and incubated overnight at 4 °C.

Competent *E.coli* DH1 cells were transformed with 1 µL and 10 µL aliquots of the ligation mix (Invitrogen, San Diego, CA) according to the manufacturer's directions. Ampicillin resistant colonies were obtained on LB plates containing 100 µg/mL ampicillin. Selected clones grown in 2.0 mL overnight cultures were prepared, samples of plasmid DNA were digested with *Hind* III and *Bam* HI separately, and a correct representative clone selected.

The resulting plasmid pSV2neo-101 was verified by size mapping and the lack of digestion with *Hind* III.

A sample of DNA from pSV2neo-10 mini-lysate was prepared by digesting with 50 units of *Bam* HI at 37°C for 2 hours. The linearized plasmid was purified from a 4 percent DNA polyacrylamide gel by electroelution. The DNA ends were made blunt by filling in the *Bam* HI site using dNTPs and Klenow fragment, as described earlier for the *Hind* III site of pSV2 neo-101.

A polylinker segment containing multiple cloning sites was incorporated at the *Bam* HI site of pSV2neo-101 to create pSV2neo-102. Equimolar amounts of two oligonucleotides, CH(+) and CH(-) (shown in Figure 14) were annealed by heating for 3 minutes at 90 °C and

cooling to 50 °C. Annealed linker DNA and blunt ended pSV2neo-101 were added, in a 40:1 molar volume to a standard 20 µL ligation reaction. *E.coli* DH1 was transformed with 0.5 µL and 5 µL aliquots of the ligation mixture (Invitrogen). Twelve ampicillin resistant colonies were selected for analysis of plasmid DNA to determine whether the linker had been incorporated.

A *Hind* III digest of mini-lysate plasmid DNA revealed linker incorporation in six of the clones. The plasmid DNA from several clones was sequenced, to determine the number of linker units that were blunt-end ligated to pSV2neo-101 as well as the relative orientation(s) with the linker. Clones for sequencing were selected on the basis of positive digestion with *Hind* III.

A Sequenase™ sequencing kit (United States B)iochemical Corp, Cleveland, OH was used to sequecne the DNA. A primer, NEO102SEQ, was used for sequencing and is shown in Figure 15. It is complementary to a sequence located upstream from the *Bam*HI site in the vector. Between 3 µg and 5 µg of plasmid DNA isolated from *E.coli* mini-lysates were used for sequencing. The DNA was denatured and precipitated prior to annealing, as according to the manufacturer's instructions. Electrophoresis was carried out at 1500 volts; gels were dried prior to exposure to Kodak X-ray film. Data was processed using Hitachi's DNASIS™ computer program.

From the DNA sequence data of 4 clones analyzed (see photograph of autoradiogram - Figure 16), compared to the expected sequence in Figure 14, two clones having

the desired orientationwere obtained. A representative clone was selected and designated pSV2neo-102.

A human Cκ gene was inserted into pSV2neo-102 to form pRL1000. The human Cκ DNA was contained in a 5.0 kb *Hind* III-*Bam* HI fragment (Hieter *et al.* (1980), Cell, 22:197-207).

A 3 µg sample of DNA from a mini-lysate of pSV2neo-102 was digested with *Bam* HI and *Hind* III. The vector DNA was separated from the small *Bam* HI-*Hind* III linker fragment, generated in the reaction, by electrophoresis on a 3.75 percent DNA polyacrylamide gel. The desired DNA fragment was recovered by electroelution. A pBR322 clone containing the 5.0 kb *Hind* III-*Bam* HI fragment of the human Cκ gene (see Hieter *et al.*, *supra*) was designated phumCκ. The 5.0 kb *Hind* III-*Bam* HI fragment was ligated with pSV2neo-102r and introduced into *E.coli* DH1 (Invitrogen). Ampicillin resistant colonies were screened and a clone containing the human Ck gene was designated pRL1000.

Finally, pRL1000 clones were screened by testing mini-lysate plasmid DNA from *E.coli* with *Hind* III and *Bam* HI. A clone producing a plasmid which gave 2 bands, one at 5.8 Kb (representing the vector) and the other at 5.0 kb (representing the human Cκ insert) was selected. Further characterization of pRL1000 was achieved by sequencing downstream from the *Hind* III site in the intron region of the human Cκ insert. The oligonucleotide used to prime the sequencing reaction was NEO102SEQ (Figure 15). Two hundred and seventeen bases were determined (see Figure 17). A new oligonucleotide corresponding to the (-) strand near the *Hind* III site (shown in Figure 17) was synthesized so

that clones, containing the HHum4 V_L gene that were cloned into the *Cla* I and *Hind* III sites in pRL1000 (see Figure 13), could be sequenced.

A *Cla* I-*Hind* III DNA fragment containing Hum4 V_L obtained by PCR was cloned into the plasmid vector pRL1000. DNA of pRL1000 and the Hum4 V_L were treated with *Cla* I and *Hind* III and the fragments were gel purified by electrophoresis, as described earlier.

The pRL1000 DNA fragment and fragment containing Hum4 V_L gene were ligated, and the ligation mixture used to transform *E.coli* DH1 (Invitrogen), following the manufacturer's protocol. Ampicillin resistant clones were screened for the presence of the Hum4 V_L gene by restriction enzyme analysis and a representative clone designated pRL1001 (shown in Figure 18).

Four plasmids having the correct *Cla* I-*Hind* III restriction pattern were analyzed further by DNA sequencing of the insert region (see Figure 19). *Hind* III C_K(-) (shown in Figure 17), HUMLIN1(-) (shown in Figure 10), HUMLIN2(-) (shown in Figure 10) were used as the sequencing primers. Two out of the four plasmids analyzed had the expected sequence in the coding regions (Figure 19, clones 2 and 9).

Clone 2 was chosen and used for generating sufficient plasmid DNA for cell transformations and other analysis. This plasmid was used for sequencing through the Hum4 V_L, and the upstream region to the *Cla* I site. Only one change at nucleotide position 83 from a C to a G (Figure 10) was observed, compared to a

published sequence (Klobeck *et al.* (1985), supra). The DNA sequence data also indicates that the oligonucleotides used for the PCR had been correctly incorporated in the target sequence.

The Biorad Gene Pulser™ apparatus was used to transfect Sp2/0 cells with linearized plasmid DNAs containing the light or heavy chain constructs. The Hum4 V_L was introduced in Sp2/0 cells along with corresponding heavy chains by the co-transfection scheme indicated in Table 1.

Table 1

Cell Line Designation	DNA Added		
	L Chain pRL1001	H Chain p49 g1-2.3	H Chain p83 g1-2.3
MP1-44H	20 µg	15 µg	0 µg
MP1-84H	20 µg	0 µg	15 ug

A total of 8.0 X 10^6 Sp2/0 cells were washed in sterile PBS buffer (0.8 mL of 1 X 107 viable cells/mL) and held on ice for 10 minutes. DNA of pRL1001, linearized at the *Cla* I site, and the DNA of either p49 g1-2.3 or p83 g1-2.3, linearized at their respective *Nde* I sites, were added, in sterile PBS, to the cells (see protocol - Table 2) and held at 0 °C for a further 10 minutes. A single 200 volt, 960 µF electrical pulse lasting between 20 and 30 milliseconds was used for the electroporation. After holding the perturbed cells on ice for 5 minutes, 25 mL of RPMI medium with 10 percent fetal calf serum were introduced, and 1.0 mL samples aliquoted in a 24 well tissue culture plate. The cells were incubated at 37 °C in a 5 percent CO_2 atmosphere.

After 48 hours, the media was exchanged with fresh selection media, now containing both 1 mg/mL Geneticin (G418) (Difco) and 0.3 µg/ml mycophenolic acid/gpt medium. Resistant cells were cultured for 7-10 days.

Supernatants from wells having drug resistant colonies were tested on ELISA plates for activity against TAG-72. A roughly 10 percent pure TAG-72 solution prepared from LS147T tumor xenograft cells was diluted 1:40 and used to coat flexible polyvinyl chloride microtitration plates (Dynatech Laboratories, Inc.). Wells were air-dried overnight, and blocked the next day with 1 percent BSA. Supernatant samples to be tested for anti-TAG-72 antibody were added to the washed wells and incubated for between 1 and 2 hours at 37 °C. Alkaline phosphatase labeled goat anti-human IgG (diluted 1:250) (Southern Biotech Associates, Birmingham, AL) was used as the probe antibody. Incubation was for 1 hour. The substrate used was p-nitrophenylphosphate. Color development was terminated by the addition of 1.0 N NaOH. The plates were read spectrophotometrically at 405 nm and 450 nm, and the values obtained were 405 nm-450 nm.

Those samples producing high values in the assay were subcloned from the original 24 well plate onto 96 well plates. Plating was done at a cell density of half a cell per well (nominally 50 cells) to get pure monoclonal cell lines. Antibody producing cell lines were frozen down in media containing 10 percent DMSO.

Two cell lines were procured having the designations: MP1-44H and MP1-84H. MP1-44H has the chimeric CC49 γ1 heavy chain with the Hum4 V_L light

chain; and MP1-84H has the chimeric CC83 g1 heavy chain with the HumVkIV light chain.

A 1.0 L spinner culture of the cell line MP1-44H was grown at 37°C for 5 days for antibody production. The culture supernatant was obtained free of cells by centrifugation and filtration through a 0.22 micron filter apparatus. The clarified supernatant was passed over a Protein A cartridge (Nygene, New York). Immunoglobulin was eluted using 0.1 M sodium citrate buffer pH 30. The pH of the eluting fractions containing the antibody was raised to neutrality by the addition of Tris base, pH 9.0. The antibody-containing fractions were concentrated and passed over a Pharmacia Superose 12 HR 10/30 gel filtration column. A protein was judged to be homogeneous by SDS polyacrylamide gel electrophoresis. Isoelectric focusing further demonstrated the purity of MP1-44H.

The biological performance of the human composite antibody, MP1-44H, was evaluated by comparing immunohistochemistry results with two other anti-TAG-72 antibdoies CC49 (ATCC No. HB 9459) and Ch44 (ATCC No. HB 9884). Sections of human colorectal tumor embedded in paraffin were tested with the three antibodies by methods familiar to those skilled in this art. All three antibodies gave roughly equivalent binding recognition of the tumor antigen present on the tumor tissue sample.

A further test of the affinity and biological integrity of the human composite antibody MP1-44H was a competition assay, based on cross-competing radioiodine--labeled versions of the antibody with CC49 and Ch44 in all combinations. From the data shown in Figure 20, it

is apparent that the affinity of all 3 antibodies is equivalent and can bind effectively to tumor antigen.

MP1-44H (ATCC HB 10426) and MP1-84H (ATCC HB 10427) were deposited at the American Type Culture Collection (ATCC). The contract with ATCC provides for permanent availability of the cell lines to the public on the issuance of the U.S. patent describing and identifying the deposit or the publications or upon the laying open to the public of any U.S. or foreign patent application, which ever comes first, and for availability of the cell line to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 CFR §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638). The assignee of the present application has agreed that if the cell lines on deposit should die or be lost or destroyed when cultivated under suitable conditions for a period of thirty (30) years or five (5) years after the last request, it will be promptly replaced on notification with viable replacement cell lines.

Example 2

Single-chain antibodies consist of a V_L, V_H and a peptide linker joining the V_L and V_H domains to produce SCFVs. A single chain antibody, SCFV1, was constructed to have the Hum4 V_L as V Domain 1 and CC49 V_H as V Domain 2 (see Figure 21).

The polypeptide linker which joins the two V domains was encoded by the DNA introduced at the 3' end of the V_L DNA during the PCR. The oligonucleotides SCFV1a and SCFV2 were designed to obtain the DNA segment

incorporating part of the yeast invertase leader sequence, the Hum4 V_L and the SCFV linker.

The polypeptide linker for SCFV1 was encoded in oligonucleotide SCFV1b (see below). The underlined portions of the oligonucleotides SCFV1a and SCFV1b are complementary to sequences in the Hum4 V_L and linker respectively. The sequences of SCFV1a and SCFV1b are as follows, with the hybridizing sequences underlined:

SCFV1a with the *Hind* III in bold:

Hind III

5'CTGCAAGCTTCCTTTTCCTTTTGGCTGGTTTTGCAGCCAAAATATCTGCAG
ACATCGTGATGACCCAGTC-3'

SCFV1b with the *Aat* II site in bold:

5'-CGTAAGACGTCTAAGGAACGAAATTGGGCCAATTGTTCTGAGGA
GACCGAACCTGACTCCTTCACCTTGGTCCCTCCGCCG-3'

The target DNA in the PCR was pRL1001 (shown in Figure 18). The PCR was performed pursuant to the teachings of Mullis *et al. supra*. A DNA fragment containing the Hum4 V_L-linker DNA component for the construction of SCFV1 was obtained and purified by polyacrylamide gel electrophoresis according to the teachings of Sambrook *et al.*, *supra*.

p49 g1-2.3, containing CC49 V_H, was the target DNA in the PCR. PCR was performed according to the methods of Mullis *et al.*, *supra*. The oligonucleotides used for the PCR of CC49 V_H are as follows, with the hybridizing sequences underlined:

SCFV1c, with the *Aat* II site in bold:

5'-CCTTAGACGTCCAGTTGCAGCAGTCTGACGC-3'

SCFV1d, with the *Hind* III site in bold:

5'-GATCAAGCTTCACTAGGAGACGGTGACTGAGGTTCC-3'

The purified Hum4 V_L-linker and V_H DNA fragments were treated with *Aat* II (New England Biolabs, Beverly, MA) according to the manufacturer's protocol, and purified from a 5 percent polyacrylamide gel after electrophoresis. An equimolar mixture of the *Aat* II fragments was ligated overnight. The T4 DNA ligase was heat inactivated by heating the ligation reaction mixture at 65 °C for 10 minutes. Sodium chloride was added to the mixture to give a final concentration of 50 mM and the mixture was further with *Hind* III. A *Hind* III DNA fragment was isolated and purified from a 4.5 percent polyacrylamide gel and cloned into a yeast expression vector (see Carter *et al.* (1987), In: DNA

Cloning, A Practical Approach, Glover (ed.) Vol. III: 141-161). The sequence of the fragment, containing the contiguous SCFV1 construct, is set forth in Figure 22.

The anti-TAG-72 SCFV1 described herein utilized the yeast invertase leader sequence (shown as positions -19 to -1 of Figure 22), the Hum4 V_L (shown as positions 1 to 113 of Figure 22), an 18 amino acid linker (shown as positions 114 to 132 of Figure 22) and CC49 V_H (shown as positions 133 to 248 of Figure 22).

The complete DNA and amino acid sequence of SCFV1 is given in Figure 22. The oligonucleotides used to sequence the SCFV1 are set forth below.

TPI:

5'-CAATTTTTTGTTTGTATTCTTTTC-3'.

HUVKF3:

5'-CCTGACCGATTCAGTGGCAG-3'.

DC113:

5'-TCCAATCCATTCCAGGCCCTGTTCAGG-3'.

SUC2T:

5'-CTTGAACAAAGTGATAAGTC-3'.

Example 3

A plasmid, pCGS517 (Figure 23), containing a prorennin gene was digested with *Hind* III and a 6.5 kb fragment was isolated. The plasmid pCGS517 has a triosephosphate isomerase promoter, invertase [SUC2] signal sequence, the prorennin gene and a [SUC2]

terminator. The *Hind* III-digested SCFV1 insert obtained above (see Figure 23) was ligated overnight with the *Hind* III fragment of pCGS517 using T4 DNA ligase (Stratagene, La Jolla, CA).

The correct orientation existed when the *Hind* III site of the insert containing part of the invertase signal sequence ligated to the vector DNA to form a gene with a contiguous signal sequence. *E.coli* DHI (Invitrogen) cells were transformed and colonies screened using a filter-microwave technique (see Buluwela, *et al.* (1989), Nucleic Acids Research, 17:452). From a transformation plate having several hundred colonies, 3 positive clones were obtained. Digesting the candidate plasmids with *Sal* I and *Kpn* I, each a single cutter, differentiated between orientations by the size of the DNA fragments produced. A single clone, pDYSCFV1 (Figure 23), had the correct orientation and was used for further experimentation and cloning. The probe used was derived from pRL1001, which had been digested with *Kpn* I and *Cla* I (see Figure 18). The probe DNA was labeled with ^{32}P α-dCTP using a random oligonucleotide primer labeling kit (Pharmacia LKB Biotechnology, Piscataway, NJ).

The next step was to introduce the *Bgl* II-*Sal* 1 fragment from pDYSCFV1 into the same restriction sites of another vector (ca. 9 kb), which was derived from PCGS515 (Figure 23). to give an autonomously replicating plasmid in *S.cerevisiae*.

DNA from the vector and insert were digested in separate reactions with *Bgl* II and *Sal* I using 10X buffer number 3 (50 MM Tris-HCI (pH 8.0), 100 mM NaCl, BRL). The DNA fragment from pDYSCFV1 was run

in and electroeluted from a 5 percent polyacrylamide gel and the insert DNA was run and electroeluted from a 3.75 percent polyacrylamide gel. A standard ligation using T4 DNA ligase (Stratagene) and a transformation using *E. coli* DH1 (Invitrogen) was carried out. Out of 6 clones selected for screening with *Bgl* II and *Sal* II, all 6 were correctly oriented, and one was designated pCGS515/SCFV1 (Figure 23).

DNA sequencing of pCGS515/SCFVI DNA was done using a Sequenase™ kit (U.S. Biochemical, Cleveland, OH) using pCGS515/SCFV1 DNA. The results have been presented in Figure 22 and confirm the sequence expected, based on the linker, the Hum4 V_L and the CC49 V_H.

Transformation of yeast cells using the autonomosly replicating plasmid pCGS515/SCFV1 was carried out using the lithium acetate procedures described in Ito *et al.* (1983), J. Bacteriol., 153:163-168; and Treco (1987), In: Curent Protocols in Molecular Biology, Ausebel *et al.* (eds), 2:13.71-13.7.6. The recipient strain of *S.cerevisiae* was CGY1284 having the genctype - MAT α (mating strain α), ura 3-52 (uracil auxotrophy), SSC1-1 (supersecreting 1), and $PEP4^+$ (peptidase 4 positive).

Transformed clones of CGY1284 carrying SCFV plasmids were selected by their ability to grow on minimal media in the absence of uracil. Transformed colonies appeared within 3 to 5 days. The colonies were transferred, grown and plated in YEPD medium. Shake flasks were used to provide culture supernatant with expressed product.

An ELISA procedure was used to detect biological activity of the SCFV1. The assay was set up such that the SCFV would compete with biotinylated CC49 (biotin-CC49) for binding to the TAG-72 antigen on the ELISA plate .

SCFV1 protein was partially purified from a crude yeast culture supernatant, using a Superose 12 gel filtration column (Pharmacia LKB Biotechnology), and found to compete with biotinylated CC49 in the competition ELISA. These results demonstrate that the SCFV1 had TAG-72 binding activity.

The SCFV1 protein has been detected by a standard Western protocol (see Towbin *et al.* (1979), Proc. Natl. Acad. Sci., U.S.A., 76:4350-4354). The detecting agent was biotinylated FAID14 (ATCC No. CRL 10256), an anti-idiotypic monoclonal antibody prepared from mice that had been immunized with CC49. A band was visualized that had an apparent molecular weight of approximately 26,000 daltons, the expected size of the SCFV1. This result demonstrated that the SCFV1 had been secreted and properly processed.

Example 4

The following example demonstrates the cloning of human V_H genes into a SCFV plasmid construct containing sequence coding for the Hum4 V_L and a 25 amino acid linker called UNIHOPE.

A vector was prepared from plasmid pRW 83 containing a chloramphenicol resistance (Cam^r) gene for clone selection, and a *penP* gene with a *penP* promoter and terminator (see Mezes, *et al.* (1983), J. Biol. Chem., 258:11211-11218) and the *pel B* signal sequence (see Lei

et al. (1987) *supra*). The vector was designated Fragment A. (see Figure 24). The *penP* gene was removed with a *Hind* III/*Sal* I digest.

The *penP* promoter and *pel B* signal sequence were obtained by a PCR using pRW 83 as a template and oligonucleotides penP1 and penP2 as primers. The fragment was designated Fragment B (see Figure 24). A *Nco* I enzyme restriction site was introduced at the 3' end of the signal sequence region by the penP2 oligonucleotide.

penP1:

5'-CGATAAGCTTGAATTCCATCACTTCC-3'

penP2:

5'-GGCCATGGCTGGTTGGGCAGCGAGTAATAACAATCCAGCG GCT GCCGTAGGCAATAGGTATTTCATCAAAATCGTCTCCCTCCGTTTGAA-3'

A SCFV comprised of a Hum4 V_L, a CC49 V_H, and an 18 amino acid linker (Lys Glu Ser Gly Ser Val Ser Ser Glu Gln Leu Ala Gln Phe Arg Ser Leu Asp) was obtained from pCGS515/SCFV1 by PCR using oligonucleotides penP3 and penP6. This fragment was designated Fragment D (see Figure 24). A *Bcl* I site was introduced at the 3' end of the V_H region by the penP6 oligonucleotide.

penP3:

5'-GCTGCCCAACCAGCCATGGCCGACATCGTGATGACCCAGTCTCC-3'

penP6(-):

5'-CTCTTGATCACCAAGTGACTTTATGTAAGATGATGTTTTG ACG GATTCATCGCAATGTTTTTATTTGCCGGAGACGGTGACTGAGGTTCC-3'

Fragments B and D were joined by PCR using oligonucleotides penP1 and penP6, following the

procedures of Horton *et al.*, *supra*. The new fragment was designated E (See Figure 24).

Fragment C containing the *penP* termination codon was isolated by digesting pRW 83 with *Bcl* I and *Sal* I, and designated Fragment C. pRW 83 was isolated from *E. coli* strain GM161, which is DNA methylase minus or dam$^-$.

Plasmid pSCFV 31 (see Figure 24) was created with a three part ligation Fragments A, C, and E.

The *Nco* I restriction enzyme site within the Cam^r gene and the *Hin*d III site located at the 5' end of the *penP* promoter in pSCFV 31 were destroyed through a PCR DNA amplification using oligonucleotides Nco1.1 and Nco1.3(-) to generate an *Eco* RI-*Nco* I fragment and oligonucleotides Nco1.2 and Nco1.4c(-) to generate a *Nco* I to *Eco* RI fragment. These two fragments were joined by PCR-SOE using oligonucleotides Nco1.1 and Nco1.4c(-). The oligonucleotides are set forth below:

Nco1.1:

5'-TCCGGAATTCCGTATGGCAATGA-3'

Nco1.3(-):

5'-CTTGCGTATAATATTTGCCCATCGTGAAAACGGGGGC-3'

Nco1.2:

5'-ATGGGCAAATATTATACGCAAG-3'

Nco1.4c(-):

5'-CACTGAATTCATCGATGATAAGCTGTCAAACATGAG-3'

pSCFV 31 was digested with *Eco* RI and the larger fragment was isolated by polyacrylamide gel electrophoresis. To prevent self ligation, the DNA was

dephosphorylated using calf intertinal alkaline phosphatase according to the teachings of Sambrook *et al.*, *supra*.

A two part ligation of the larger pSCFV 31 digested fragment and the PCR-SOE fragment, described above, resulted in the creation of pSCFV 31b (see Figure 25).

pSCFV 31b was digested with *Nco* I and *Sal* I and a fragment containing the Cam^r gene was isolated.

The Hum4 V_L was obtained by PCR DNA amplification using pCGS515/SCFV1 as a template and oligonucleotides 104BH1 and 104BH2(-) as primers.

104BH1:

5'-CAGCCATGGCCGACATCGTGATGACCCAGTCTCCA-3'

104BH2(-):

5'-AAGCTTGCCCCATGCTGCTTTAACGTTAGTTTTATCTGCTGG AGACAGAGTGCCTTCTGCCTCCACCTTGGTCCCTCCGCCGAAAG-3'

The CC49 V_H was obtained by PCR using p49 g1-2.3 (Figure 5) as a template and oligonucleotides 104B3 and 104B4(-) as primers. A *Nhe* I enzyme restriction site was introduced just past the termination codon in the 3' end (before the *Bcl* I site) by oligonucleotide 104B4(-).

104B3:

5'-GTTAAAGCAGCATGGGGCAAGCTTATGACTCAGTTGCAGCAGTCTGACGC-3'

104B4(-):

5'-CTCTTGATCACCAAGTGACTTTATGTAAGATGATGTTTTGACGGATT
CATCGCTAGCTTTTTATTTGCCATAATAAGGGGAGACGGTGACTGAGGTTCC-3'

In the PCR which joined these two fragments using oligonucleotides 104BH1 and 104B4(-) as primers, a coding region for a 22 amino acid linker was formed.

A fragment C (same as above) containing the *penP* termination codon was isolated from pRW 83 digested with *Bcl* I and *Sal* I.

Plasmid pSCFV 33H (see figure 25) was created with a three part ligation of the vector, fragment C, and the SCFV fragment described above.

pSCFV 33H was digested with *Nco*I and *Nhe*I, and the DNA fragment containing the Cam^r gene was isolated as a vector.

Hum4 V_L was obtained by PCR DNA amplification using pRL1001 (see Figure 18) as a template and oligonucleotides UNIH1 and UNIH2(-) as primers. Oligonucleotides for the PCR were:

UNIH1:

5'-CAGCCATGGCCGACATTGTGATGTCACAGTCTCC-3'

The *Nco* I site is in bold and the hybridizing sequence is underlined.

UNIH2(-):

5'-GAGGTCCGTAAGATCTGCCTCGCTACCTAGCAAA
AGGTCCTCAAGCTTGATCACCACCTTGGTCCCTCCGC-3'

The *Hind* III site is in bold.

The CC49 V_H was obtained by a PCR using p49 g1-2.3 (see Figure 6) as a template and oligonucleotides UNI3 and UNI4(-) as primers.

UNI3:

5'-AGCGAGGCAGATCTTACGGACCTCGAGGTTCAGTTGCAGCAGTCTGAC-3'.

The *Xho* I site is in bold and the hybridizing sequence is underlined.

UNI4(-):

5'-CATCGCTAGCTTTTTATGAGGAGACGGTGACTGAGGTTCC-3'.

The *Nhe* I site is in bold and the hybridizing sequence is underlined.

Oligonucleotides UNIH1 and UNI4(-) were used in the PCR-SOE amplification which joined the Hum4 V_L and CC49 V_H fragments and formed a coding region for a negatively charged fifteen amino acid linker. The DNA was digested with *Nhe* I and *Nco* I and ligated with the vector fragment from the *Nco* I-*Nhe* I digest of pSCFV 33H. The resultant plasmid was designated pSCFV UNIH (shown in Figure 25).

With the construction of pSCFV UNIH, a universal vector for any SCFV was created with all the desired restriction enzyme sites in place.

pSCFV UNIH was digested with *Hind* III/*Xho* I, and the large DNA fragment containing the Cam^r gene. Hum4 V_L and CC49 V_H was isolated.

A fragment coding for a 25 amino acid linker, was made by annealing the two oligonucleotides shown below. The linker UNIHOPE is based on 205C SCA™ linker (see Whitlow, (1990) Antibody Engineering: New Technology and Application Implications. IBC USA

Conferences Inc, MA), but the first amino acid was changed from serine to leucine and the twenty-fifth amino acid were was changed from glycine to leucine, to accomodate the *Hind* III and *Xho* I restriction sites. The nucleotide sequence encoding the linker UNIHOPE is set forth below:

UNIHOPE (Figure 26):

5'-TATAAAGCTTAGTGCGGACGATGCGAAAAAGGATGCTGCGAAG
AAGGATGACGCTAAGAAAGACGATGCTAAAAAGGACCTCGAGTCTA-3'

UNIHOPE(-) (Figure 26):

5'TAGACTCGAGGTCCTTTTTAGCATCGTCTTTCTTAGCGT CAT
CCTTCTTCGCAGCATCCTTTTTCGCATCGTCCGCACTAAGCTTTATA-3'

The resulting strand was digested with *Hind* III/*Xho* I and ligated into the vector, thus generating the plasmid pSCFV UHH (shown in Figure 27). Plasmid pSCFV UHH expresses a biologically active, TAG-72 binding SCFV consisting of the Hum4 V_L and CC49 V_H. The expression plasmid utilizes the β-lactamase *penP* promoter, pectate lyase *pel*B signal sequence and the *penP* terminator region. Different immunoglobulin light chain variable regions can be inserted in the *Nco* I-*Hind* III restriction sites, different SCFV linkers can be inserted in the *Hind* III-*Xho* I sites and different immunoglobulin heavy chain variable regions can be inserted in the *Xho* I-*Nhe* I sites.

E.coli AG1 (Stratagene) was transformed with the ligation mix, and after screening, a single chloramphenicol resistant clone, having DNA with the correct restriction map, was used for further work.

The DNA sequence and deduced amino acid sequence of the SCFV gene in the resulting plasmid are shown in Figure 26.

E.coli AG1 containing pSCFV UHH were grown in 2 ml of LB broth with 20 µg/mL chloramphenicol (CAM 20). The culture was sonicated and assayed using a competition ELISA. The cells were found to produce anti-TAG-72 binding material. The competition assay was set up as follows: a 96 well plate was derivatized with a TAG-72 preparation from LS174T cells. The plate was blocked with 1% BSA in PBS for 1 hour at 31 °C and then washed 3 times with 200 µL of biotinylated CC49 (1/20,000 dilution of a 1 mg/mL solution) were added to the wells and the plate was incubated for 30 minutes at 31 °C. The relative amounts of TAG-72 bound to the plate, biotinylated CC49, streptavidin-alkaline phosphatase, and color development times were determined empirically in order not to have excess of either antigen or biotinylated CC49, yet have enough signal to detect competition by SCFV. Positive controls were CC49 at 5 µg/mL and CC49 Fab at 10 µL/mL. Negative controls were 1% BSA in PBS and/or concentrated LB. Unbound proteins were washed away.

Fifty microliters of a 1:1000 dilution of streptavidin conjugated with alkaline phosphatase (Southern Biotechnology Associates, Inc., Birmingham, AL) were added and the plate was incubated for 30 minutes at 31 °C. The plate was washed 3 more times. Fifty microliters of a para-nitrophenylphosphate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were added and the color reaction was allowed to develop for a minimum of 20 minutes. The relative amount of SCFV binding was measured by optical density scanning at 405-450 nm using a microplate reader (Molecular Devices Corporation, Menlo Park, CA). Binding of the SCFV resulted in decreased binding of the

biotinylated CC49 with a concomitant decrease in color development. The average value for triplicate test samples is shown in the table below:

Sample (50 µL) (mixed 1:1 with CC49 Biotin)	OD 405 nm - OD 450 nm Value at 50 minutes
Sonicate *E.coli* AG1/ pSCFVUHH clone 10	0.072
Sonicate *E.coli* AG1/ pSCFVUHH clone 11	0.085
CC49 at 5 mg/mL	0.076
CC49 Fab at 10 mg/mL	0.078
LB (negative control)	0.359

The data indicates that there was anti-TAG-72 activity present in the *E.coli* AGI/pSCFVUHH clone sonicate.

Example 7

The plasmid pSCFVUHH may be used to host other V_H genes on *Xho* I-*Nhe* I fragments and test in a SCFV format, following the procedures set forth below. A schematic for this process is shown here.

Discovery of Hum4 V_L-V_H combinations that compete with known prototype TAG-binding antibodies or mimetics.

pSCFVUHH *Xho* I/*Nhe* I
Vector DNA Fragment
(CC49 V_H removed)
or pATDFLAG XhoI/NheI Vector DNA Fragment

Isolate mRNA from peripheral blood lymphocytes

↓

Synthesize cDNA

↓

PCR amplify human V_H genes using oligos HVH135, HVH2A, HVH46 (as the 5' targeting oligos) and JH1245, JH3 and JH6 (as the 3' targeting oligos) in all 9 combinations.

↓

Gel purify DNA

↓

Digest with *Xho* I and *Nhe* I

↓

Gel purify DNA (V_H inserts)

Ligate Vector and V_H insert DNAs → Transform *E.coli*

↓

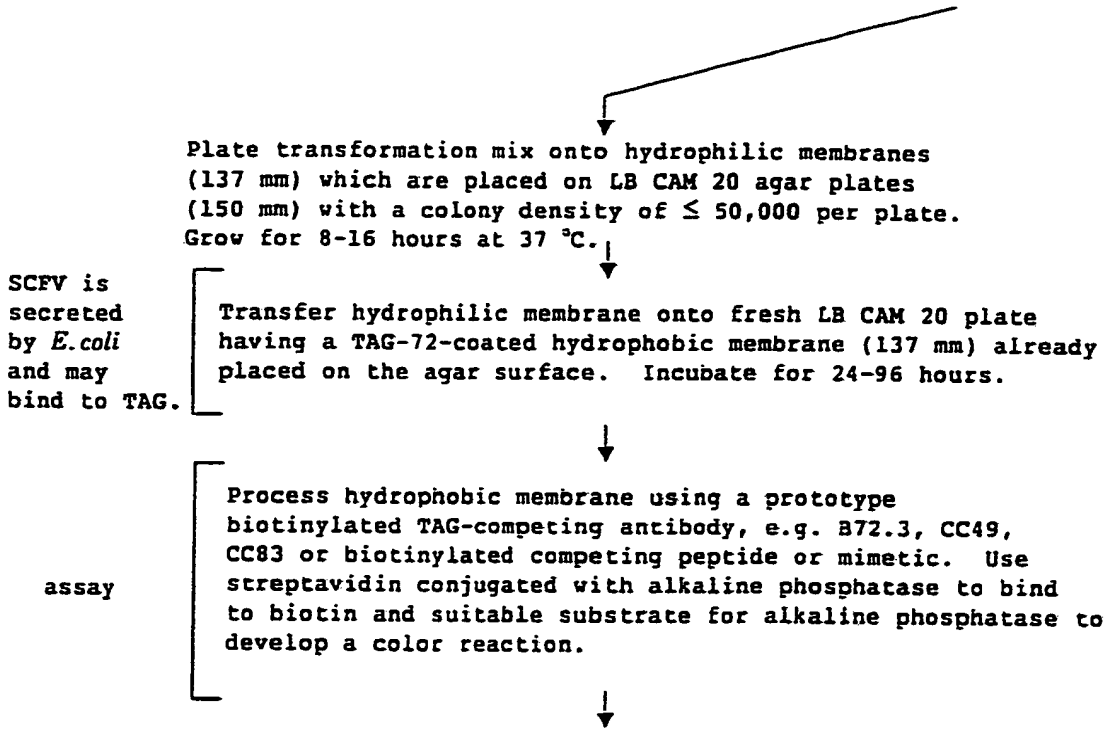


Plate transformation mix onto hydrophilic membranes (137 mm) which are placed on LB CAM 20 agar plates (150 mm) with a colony density of ≤ 50,000 per plate. Grow for 8-16 hours at 37 °C.
SCFV is secreted by E.coli and may bind to TAG.
Transfer hydrophilic membrane onto fresh LB CAM 20 plate having a TAG-72-coated hydrophobic membrane (137 mm) already placed on the agar surface. Incubate for 24-96 hours.
assay
Process hydrophobic membrane using a prototype biotinylated TAG-competing antibody, e.g. B72.3, CC49, CC83 or biotinylated competing peptide or mimetic. Use streptavidin conjugated with alkaline phosphatase to bind to biotin and suitable substrate for alkaline phosphatase to develop a color reaction.
Co-relate clear zones on membrane assay with colony(ies) on hydrophilic membrane. Isolate/purify correct clone as necessary. Characterize DNA (sequence) and determine binding affinity of SCFV to TAG-72. Purify SCFV and perform in vivo animal biodistribution studies.
Determine normal:tumor tissue binding profile by immunohistochemistry.
Utilize Hum4 V_L and V_H in preferred antibody formats e.g. whole Ig (IgG1, IgE, IgM etc.) Fab or F(ab')$_2$ fragment, or SCFV.

Isolating total RNA from peripheral blood lymphocytes:

Blood from a normal, healthy donor is drawn into three 5 mL purple-cap Vacutainer tubes. Seven mL of blood are added to two 15 mL polypropylene tubes. An equal volume of lymphoprep (cat# AN5501, Accurate) is added and the solution is mixed by inversion. Both tubes are centrifuged at 1000 rpm and 18 °C for 20 minutes. The resulting white area near the top of the liquid (area not containing red blood cells) is removed from each sample and placed into two sterile polypropylene centrifuge tube. Ten mL of sterile PBS are added and the tube mixed by inversion. The samples are centrifuged at 1500 rpm and 18 °C for 20 minutes Total RNA is isolated from resulting pellet according to the RNAzol B Method (Chomczynski and Sacchi (1987), Analytical Biochemistry, 162:156-159). Briefly, the cell pellets are lysed in 0.4 mL RNAzol solution (cat#:CS-105, Cinna/Biotecx). RNA is solubilized by passing the cell pellet through a 1 mL pipet tip. Sixty µL of chloroform are added and the solution is shaken for 15 seconds. RNA solutions are then placed on ice for 5 minutes. Phases are separated by centrifugation at 12000 x g and 4 °C for 15 minutes. The upper (aqueous) phases are transferred to fresh RNase-free microcentrifuge tubes. One volume of isopropanol is added and the samples placed at -20 °C for 1 hour. The samples are then placed on dry ice for 5 minutes and finally centrifuged for 40 seconds at 14,000 x g and 4 °C. The resulting supernatant is removed from each sample and the pellet is dissolved in 144 µL of sterile RNase-free water. Final molarity is brought to 0.2M NaCL. The DNA is reprecipitated by adding 2 volumes of 100% ethanol, leaving on dry ice for 10 minutes, and

centrifugation at 14,000 rpm and 4 °C for 15 minutes. The supernatants are then removed, the pellets washed with 75% ethanol and centrifuged for 8 minutes at 12000 x g and 4 °C. The ethanol is then removed and the pellets dried under vacuum. The resulting RNA is then dissolved in 20 sterile water containing 1 µl RNasin (cat#:N2511, Promega).

cDNA synthesis:

cDNA synthesis is performed using a Gene Amp™ PCR kit (cat#: N808-0017 Perkin Elmer Cetus), RNasin™ (cat#: N2511, Promega), and AMV reverse transcriptase (cat#: M9004, Promega). The following protocol is used for each sample:

Components	Amount
$MgCl_2$ solution	4 µl
10 µl PCR buffer II	2 µl
dATP	2 µl
dCTP	2 µl
dGTP	2 µl
dTTP	2 µl
3' primer (random hexamers)	1 µl
RNA sample	2 µl
RNasin	1 µl
AMV RT	1.5 µl

Samples are heated at 80 °C for 3 minutes then slowly cooled to 48 °C. The samples are then centrifuged for 10 seconds. AMV reverse transcriptase is added to the samples which are then incubated for 30 minutes at 37 °C. After incubation, 0.5 µl of each dNTP

and 0.75 reverse transcriptase (cat#:109118, Boehringer Mannheim) are added. The samples are incubated for an additional 15 minutes at 37 °C.

PCR Reaction:

Oligonucleotides are designed to amplify human V_H genes by polymerase chain reaction. The 5' oligonucleotides are set forth below:

HVH 135:
5'-TATTCTCGAGGTGCA(AG)CTG(CG)TG(CG)AGTCTGG-3'

HVH2A:
5'-TATTCTCGAGGTCAA(CG)TT(AG)A(AG)GGAGTCTGG-3'

HVH46:
5'-TATTCTCGAGGTACAGCT(AG)CAG(CG)(AT)GTC(ACG)GG-3'

The 3' oligonucleotides are set forth below:

JH1245:
5'-TTATGCTAGCTGAGGAGAC(AG)GTGACCAGGG-3'

JH3:
5'-TTATGCTAGCTGAAGAGACGGTGACCATTG

JH6:
5'-TTATGCTAGCTGAGGAGACGGTGACCGTGG-3'

PCR reactions are performed with a GeneAmp™ PCR kit (cat#:N808-0017, Perkin Elmer Cetus). Components are listed below:

Components	Amount
ddH_2O	75 µl
10 x buffer	10 µl
dATP	2 µl
dCTP	2 µl
dGTP	2 µl
dTTP	2 µl
1* Target DNA	1 µl
2* 5' primer	2.5 µl
3' primer	2.0 µl
3* AmpliTaq™ Polymerase	1.3 µl

*components added in order at 92 °C of first cycle

PCR program: step 1 94 °C for 30 seconds

step 2 60 °C for 1 minutes

step 3 72 °C for 45 seconds

Approximately 35 cycles are completed for each reaction. All PCR reactions are performed using a Perkin Elmer Cetus PCR System 9600 thermal cycler.

Treatment of Human V_H inserts with *Xho* I and *Nhe* I:

Human V_H genes are digested with *Xho* I (cat#: 131L, New England Biolabs) and *Nhe* I (cat#: 146L, New England Biolabs). The following protocol is used for each sample:

SUBSTANCE	AMOUNT
DNA	20 µl
NEB Buffer #2	4.5 µl
Nhe I	2 µl
Xho I	2 µl
ddH_2O	16.5 µl

Samples are incubated at 37 °C for one hour. After this incubation, an additional 1.5 µL *Nhe* I is added and samples are incubated an additional two hours at 37 °C.

Purification of DNA:

After the restrictive enzyme digest, DNA is run on a 5 percent polyacrylamide gel (Sambrook *et al.* (1989), *supra*). Bands of 390-420 bp in size are excised from the gel. DNA is electroeluted and ethanol precipitated according to standard procedures.

PCR products resulting from oligonucleotide combinations are pooled together: JH1245 with HVH135, HVH2A and HVH46; JH3 with HVH135, HVH2A and HVH46; JH6 with HVH135, HVH2A and HVH46. The volume of the resulting pools are reduced under vacuum to 50 microliters. The pools are then purified from a 4 percent polyacrylamide gel (Sambrook *et al.* (1989), *supra*) to isolate DNA fragments. Bands resulting at 390-420 bp are excised from the gel. The DNA from excised gel slices is electroeluted according to standard protocols set forth in Sambrook, *supra*.

Isolation of pSCFVUHH *Xho* I/*Nhe* I Vector Fragment

Approximately 5 µg in 15 µL of pSCFVUHH plasmid is isolated using the Magic Mini-prep™ system (Promega). To this is added 5.4 µL OF 10X Buffer #2 (New England Biolabs), 45 units of *Xho* I (New England Biolabs), 15 units of *Nhe* I and 24 µL of ddH_2O. The reaction is allowed to proceed for 1 hour at 37 °C. The sample is loaded on a 4% polyacrylamide gel, electrophoresed and purified by electroelution, as described earlier. The DNA pellet is dissolved in 20 µL of ddH_2O.

One hundred nanograms of pSCFVUHH digested with *Xho* I/*Nhe* I is ligated with a 1:1 molar ratio of purified human V_H inserts digested with *Xho* I and *Nhe* I using T4 DNA ligase (Stratagene). Aliquots are used to transform competent *E. coli* AG1 cells (Stratagene) according to the supplier's instructions.

GVWP hydrophilic membranes (cat# GVWP14250, Millipore) are placed on CAM 20 LB agar plates (Sambrook *et al.*, 1989). One membrane is added to each plate. Four hundred microliters of the *E. coli* AG1 transformation suspension from above are evenly spread over the surface of each membrane. The plates are incubated for 16 hours at 37 °C ambient temperatures.

Preparation of TAG-72-coated membranes:

A 1% dilution of partially purified tumor associated glycoprotein-72 (TAG-72) produced in LS174 T-cells is prepared in TBS (cat# 28376, Pierce). Ten milliliters of the TAG dilution are placed in a petri plate (cat# 8-757-14, Fisher) for future use. Immobilon-P PVDF transfer membranes (cat# SE151103, Millipore) are immersed in methanol. The membranes are

than rinsed three times in sterile double distilled water. After the final wash, the excess water is allowed to drain. Each of the membranes are placed in 10 milliliters of dilute TAG-72. The membranes are incubated at ambient temperature from 1 hour with gentle shaking. After incubation, the membranes are blocked with Western blocking solution (25 mM Tris, 0.15 M NaCl, pH 7.6; 1% BSA) for about 1 hour at ambient temperature.

Blocking solution is drained from the TAG membranes. With the side exposed to TAG-72 facing up, the membranes are placed onto fresh CAM 20 plates. Resulting air pockets are removed. The bacterial membranes are then added, colony side up, to a TAG membrane. The agar plates are incubated for 24 to 96 hours at ambient temperatures.

The orientation of the TAG-72 and bacterial membranes are marked with permanent ink. Both membranes are removed from the agar surface. The TAG-72 membrane is placed in 20 ml of Western antibody buffer (TBS in 0.05% Tween-20, cat# P-1379, Sigma Chemical Co.; 1% BSA, cat#3203, Biocell Laboratories) containing 0.2 ng of CC49-Biotin probe antibody. The bacterial membranes are replaced on the agar surface in their original orientation and set aside. CC49-Biotin is allowed to bind to the TAG membranes for 1 hour at 31 °C with gentle shaking. The membranes are then washed three times with TTBS (TBS, 0.05% Tween-20) for 5 minutes on an orbital shaker at 300 rpm. Streptavidin alkaline phosphatase (cat# 7100-04, Southern Biotechnology Associates) is added to Western antibody buffer to produce a 0.1% solution. The TAG-72 membranes are each immersed in 16 milliliters of the streptavidin solution and allowed to incubate for 30 minutes at 31 °C with

gentle shaking. After incubation, the membranes are washed as previously described. A final wash is then performed using Western alkaline phosphate buffer (8.4 g $NaCO_3$, 0.203 g $MgCl_2$-H_2O, pH 9.8), for 2 minutes at 200 rpm at ambient temperature. To develop the membranes, Western blue stabilized substrate (cat# S384B, Promega) is added to each membrane surface. After 30 minutes at ambient temperatures, development of the membranes is stopped by rinsing the membranes three times with ddH_2O. The membranes are then photographed. The membranes are then photograhed and clear zones are corelated with colonies on the hydrophilic membrane, set aside earlier. Colony(ies) are isolated for growth in culture and used to prepare plasmid DNA for sequencing and protein preparation to evaluate specificity and affinity.

Identification of Hum4 V_L, human V_H combinations using pATDFLAG.

In a second assay system, Hum4 V_L - human V_H combinations are discovered that bind to TAG-72 according to the schematic, *supra*, except for the following: at the assay step, IBI MII antibody is used as a probe to detect any Hum4 V_L - V_H SCFV combinations that have bound to the hydrophobic membrane coated with TAG-72.

The plasmid pATDFLAG was generated from pSCFVUHH (see Figure 29) to incorporate a flag-coating sequence 3' of any human V_H genes to be expressed continguously with Hum4 V_L. The plasmid pATDFLAG, when digested with *Xho* I and *Nhe* I and purified becomes the human C_H discovery plasmid containing Hum4 V_L in this SCFV format. The plasmid pATDFLAG was generated as follows. Plasmid pSCFVUHH treated with *Xho* I and *Nhe* I (isolated and described above) was used in a ligation

reaction with the annealed FLAG and FLAGNC oligonucleotides.

FLAGC:
5'-TCGAGACAATGTCGCTAGCGACTACAAGGACGATGATGACAAATAAAAAC-3'

FLAGNC:
5'-CTAGGTTTTTATTTGTCATCATCGTCCTTGTAGTCGCTAGCGACATTGTC-3'

Equimolar amounts (1 x 10^{-10} moles of each of the oligonucleotides FLAGC and FLAGNC were mixed together using a ligation buffer (Stratagene). The sample is heated to 94 °C and is allowed to cool to below 35 °C before use in the ligation reaction below.

Ligation Reaction to Obtain pATDFLAG

COMPONENT	AMOUNT
pSCFVUHH *Xho* I/*Nhe* I vector	1.5 µl
ANNEALED FLAGC/FLAGNC	0.85 µl
10X Ligation buffer	2 µl
T4 DNA LIGASE	1 µl
10 MM ATP	2 µl
ddH_2O	12.65 µl

The reaction is carried out using the following components and amounts according the ligation protocol disclosed above. *E coli* AG1 cells (Stratagene) are transformed with 3 µl of the above ligation reaction and colonies selected using CAM 20 plates. Clones having

appropriate *Nhe* I, *Xho* I and *Nhe* I/*Xho* I restriction patterns are selected for DNA sequencing.

The oligonucleotide used to verify the sequence of the FLAG linker in PATDFLAG (see Figure 28) is called PENPTSEQ: 5'-CTTTATGTAAGATGATGTTTTG-3. This oligonucleotide is derived from the non-coding strand of the *penP* terminator region. DNA sequencing is performed using Sequenase™ sequencing kit (U.S. Biochemical, Cleveland, OH) following the manufacturer's directions. The DNA and deduced amino acid sequences of the Hum4 V_L - UNIHOPE linker - FLAG peptide is shown in Figure 28.

Generating pSC49FLAG

The CC49V_H is inserted into the sites of *Xho* I - *Nhe* I pATDFLAG (see Figure 29) and evaluated for biological activity with the purpose of serving as a positive control for the FLAG assay system to detect binding to TAG-72. The new plasmid, called pSC49FLAG (see Figure 29) is generated as follows. The plasmid pATDFLAG (5 mg, purified from a 2.5 ml culture by the Magic Miniprep™ system (Promega) is treated with *Xho* I and *Nhe* I and the large vector fragment purified as described above for pSCFVUHH. The CC49 V_H insert DNA fragment is obtained by PCR amplification from pSCFVUHH and oligonucleotides UNI3 as the 5' end oligonucleotide and SC49FLAG as the 3' end oligonucleotide. The resulting DNA and amino acid sequences of this SCFV antibody, with the FLAG peptide at the C-terminus, is shown in Figure 30. The PCR reaction is carried out using 100 pmol each of the oligonucleotides, 0.1 ng of pSCFVUHH target DNA (uncut) and the standard protocol and reagents provided by Perkin Elmer Cetus. The DNA is first gel purfied, then treated with *Xho* I and *Nhe* I to

generate sticky ends and purified from a 4% polyacrylamide gel and electroeluted as described earlier. The DNA vector (pATDFLAG treated with *Xho* I and *Nhe* I) and the insert (CC49 V_H PCR product from pSCFVUHH treated with *Xho* I and *Nhe* I) are ligated in a 1:1 molar ratio, using 100 ng vector DNA (Stratagene kit) and used to transform *E.coli* AG1 competent cells (Stratagene) according to the manufacturer's directions. A colony with the correct plasmid DNA is picked as the pSC49FLAG clone.

Ligation of pATDFLAG Vector with PCR Amplified Hum4 V_H Inserts

The protocol for the ligation reaction is as follows:

COMPONENT	AMOUNT
DNA vector:pATDFLAG *Xho* I/*Nhe* I	2.5 µL
Hum V_H (X) DNA inserts: *Xho* I/*Nhe* I	6 µL
10 mM ATP (Stratagene)	2 µL
10X buffer (Stratagene)	2 µL
T4 DNA ligase (Stratagene)	1 µL
ddH_2O	6.5 µL

DNA vector, ATP, 10X buffer and ddH_2O are combined. DNA insert and T4 DNA ligase are then added. Ligation reactions are then placed in a 4 L beaker containing H_2O at 18 °C. The temperature of the water

is gradually reduced by refrigeration at 4 °C overnight. This ligation reaction generates pHum4 V_L - hum V_H (X).

Transformation of *E.coli* AG1 with pHum4 V_L-Hum V_H (X) Ligation Mix

Transformation of pATDFLAG into competent *E.coli* AG1 cells (Stratagene, La Jolla) is achieved following the supplier's protocol.

IBI MII Anti-FLAG Antibody Plate Assay

The first three steps, preparation of TAG-coated membranes, plating of bacterial membranes, and assembly of TAG and bacterial membranes, are the same as those described in the CC49-Biotin Competition Plate Assay.

After the 24 hour incubation at ambient temperatures, the membranes are washed with TTBS three times at 250 rpm for four minutes. The MII antibody (cat# IB13010, International Biotechnologies, Inc.) is then diluted with TBS to a concentration ranging from 10.85 µg/ml to 0.03 µg/ml. Ten millilters of the diluted antibody are added to each membrane. The membranes are then incubated for 1 hour at ambient temperatures and shaken on a rotary shaker at 70 rpm. After incubation, the MII antibody is removed and the membranes are washed three times at 250 rpm and ambient temperatures for 5 minutes. The final wash is removed and 20 milliliters of a 1:2000 dilution of sheep anti-mouse horseradish peroxidase linked whole antibody (cat# NA931, Amersham) is prepared with TBS and added to each membrane. The membranes are again incubated for 1 hour at ambient temperatures and 70 rpm. Following incubation, the membranes are washed three times at 250

rpm and ambient temperature for 5 minutes each. Enzygraphic Webs (cat# IB8217051, International Biotechnologies, Inc.) are used according to develop the membranes, according to the manufacturer's instructions. The membranes are then photographed.

Instead of seeing a clear zone on the developed membrane for a positive Hum4 V_L-V_H (X) clone producing an SCFV that binds to TAG-72, (as seen with the competition screening assay) in this direct FLAG - detecting assay, a blue-purple spot is indicative of a colony producing a SCFV that has bound to the TAG-72 coated membrane. The advantage of using the FLAG system is that any Hum4 V_L - V_H SCFV combination that has bound to TAG-72 will be detected. Affinities can be measured by Scatchard analysis (Scatchard (1949), *supra*) and specificity by immunohistochemistry. These canidates could then be checked for binding to a specific epitope by using the competition assay, *supra*, and a competing antibody or mimetic, if desired.

The present invention is not to be limited in scope by the cell lines deposited since the deposited embodiment is intended astwo illustration of one aspect of the invention and all cell lines which are functionally equivalent are within the scope of the invention. Indeed, while this invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications could be made therein without departing from the spirit and scope of the appended claims.

Claims

1. A composite Hum4 V_L, V_H antibody having binding affinity for TAG-72 comprising

A. a light chain having a variable region (V_L), said V_L being encoded by a DNA sequence encoding at least a portion of a light chain variable region effectively homologous to the human Subgroup IV germline gene (Hum4 V_L); and

B. a heavy chain having a variable region (V_H), said V_H being encoded by a DNA sequence segment encoding at least a portion of a heavy chain variable region (V_H) which is capable of combining with the V_L to form a three dimensional structure having the ability to bind TAG-72.

2. The composite Hum4 VL, VH antibody of Claim 1, wherein the VL is further encoded by a human J gene segment.

3. The composite Hum4 V_L, V_H antibody according to Claim 1, wherein the V_H is encoded by a DNA sequence comprising a subsegment effectively homologous to the $V_H\alpha$TAG germline gene ($V_H\alpha$TAG).

4. The composite Hum4 V_L, V_H antibody of Claim 1, wherein the V_H is further encoded by an animal D gene segment and an animal J gene segment.

5. The composite Hum4 V_L, V_H antibody of Claim 1, wherein the variable region is derived from the variable regions of CC46, CC49, CC83 or CC92.

6. The composite Hum4 V_L, V_H antibody of Claim 1, wherein the V_H comprises (1) complementarity diversity regions (CDR) being encoded by a gene derived from the $V_H\alpha$TAG. and (2) framework segments, adjacent to the CDR segments. encoded by a human genes.

7. The composite Hum4 V_L, V_H antibody of Claim 1, wherein the light chain further comprises at least a portion of a human light chain (C_L) and the heavy chain further comprises at least a portion of a animal constant region (C_H).

8. The composite Hum4 V_L, V_H antibody of Claim 6, wherein the C_H is IgG_{1-4}, IgM, IgA1, IgA2, IgD or IgE.

9. The composite Hum4 V_L, V_H antibody of Claim 7, wherein C_L is kappa or lambda.

10. A composite Hum4 V_L, V_H single chain antibody or immunoreactive fragment thereof comprising (a) a light chain having a variable region (V_L), said V_L being encoded by a DNA sequence encoding at least a portion of a light chain variable region (V_L) effectively homologous to the human Subgroup IV germline gene (Hum4 V_L); (b) a heavy chain having a variable region (V_H), said V_H being encoded by a DNA sequence segment encoding at least a portion of a heavy chain

variable region (V_H) and (c) a linker linking the V_H and V_L, wherein the polypeptide linker properly folds the V_H and V_L into a single chain antibody which is capable of forming a three dimensional structure having the ability to bind TAG-72.

11. A composite Hum4 V_L, V_H antibody conjugate comprising the composite Hum4 V_L, V_H antibody of Claims 1 through 10 conjugated to an imaging marker or a therapeutic agent.

12. The composite Hum4 V_L, V_H antibody conjugate of Claim 11, wherein the imaging marker is selected from the group consisting of ^{125}I, ^{131}I, ^{123}I, ^{111}In, ^{105}Rh, ^{153}Sm, ^{67}Cu, ^{67}Ga, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re, and ^{99m}Tc.

13. The composite Hum4 V_L, V_H antibody conjugate of Claim 11, wherein the therapeutic agent is a drug or biological response modifier, radionuclide, or toxin.

14. The composite Hum4 V_L, V_H antibody conjugate of Claim 13, wherein the drug is methotrexate, adriamycin or interferon.

15. The composite Hum4 V_L, V_H antibody conjugate of Claim 13, wherein the radionuclide is ^{131}I, ^{90}Y, ^{105}Rh, ^{47}Sc, ^{67}Cu, ^{212}Bi, ^{211}At, ^{67}Ga, ^{125}I, ^{186}Re, ^{188}Re, ^{177}Lu, ^{99m}Tc, ^{153}Sm, ^{123}I or ^{111}In.

16. A composition comprising the composite Hum4 V_L, V_H antibody of Claim 1 in a pharmaceutically acceptable, non-toxic, sterile carrier.

17. A composition comprising the composite Hum4 V_L, V_H antibodyof Claim 12 in a pharmaceutically acceptable, non-toxic, sterile carrier.

18. A composition comprising the composite Hum4 V_L, V_H antibody of Claim 13 in a pharmaceutically acceptable, non-toxic, sterile carrier.

19. A method for *in vivo* diagnosis of cancer which comprises administering to an animal a pharmaceutically effective amount of the composition of Claim 16 for the *in situ* detection of carcinoma lesions.

20. The method of Claim 19, wherein the animal is a human.

21. A method for the *in vivo* treatment of cancer which comprises administering to an animal a pharmaceutically effective amount of the composition of Claim 18.

22. The method of Claim 20, wherein the animal is a human.

23. A method for intraoperative therapy which comprises

(a) administering to an animal having at least one tumor a pharmaceutically effective amount of the composition of Claim 16, whereby the tumors are localized, and

(b) excising the tumors.

24. The method of Claim 23, wherein the animal is a human.

25. A cell capable of expressing the composite Hum4 V_L, V_H antibody or immunoreactive fragment thereof of Claim 1, said cell being transformed with

(A) a first DNA sequence encoding at least a portion of a light chain variable region (V_L) effectively homologous to the human Subgroup IV germline gene (Hum4 V_L); and

(B) a second DNA sequence encoding at least a portion of a heavy chain variable region (V_H) which is capable of combining with the V_L to form a three dimensional structure having the ability to bind TAG-72..

26. The cell of Claim 25 wherein the first and second DNA sequences are contained within at least one biologically functional expression vector.

27. A process for producing a composite Hum4 V_L, V_H antibody comprising at least the variable domains of the antibody heavy and light chains, in a single host cell, comprising the steps of:

A. transforming at least one host cell with

i) a first DNA sequence encoding at least a portion of a light chain variable region (V_L) effectively homologous to the human Subgroup IV germline gene (Hum4 V_L), and

ii) a second DNA sequence encoding at least a portion of a heavy chain variable region (V_H) which is capable of combining with the V_L to form a three dimensional structure having the ability to bind TAG-72, and

B. independently expressing said first DNA sequence and said second DNA sequence in said transformed single host cell.

28. The process according to Claim 27 wherein said first and second DNA sequences are present in at least one vector.

29. The process according to Claim 28 wherein the antibody heavy and light chains of the composite Hum4 V_L, V_H antibody are expressed in the host cell are secreted therefrom as an immunologically functional antibody molecule or antibody fragment.

30. The process of Claim 27, wherein the second DNA sequence encodes the V_H of CC46, CC49, CC83 or CC92.

31. A process for preparing an antibody or antibody fragment conjugate which comprises contacting: the composite Hum4 V_L, V_H antibody of Claim 1 with an imaging marker or therapeutic agent.

32. The process of Claim 31, wherein the imaging marker is ^{125}I, ^{131}I, ^{123}I, ^{111}In, ^{105}Rh, ^{153}Sm, ^{67}Cu, ^{67}Ga, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re or ^{99m}Tc.

33. The process of Claim 32, wherein the therapeutic agent is a radionuclide, drug or biological response modifier, toxin or another antibody.

Fig. 1

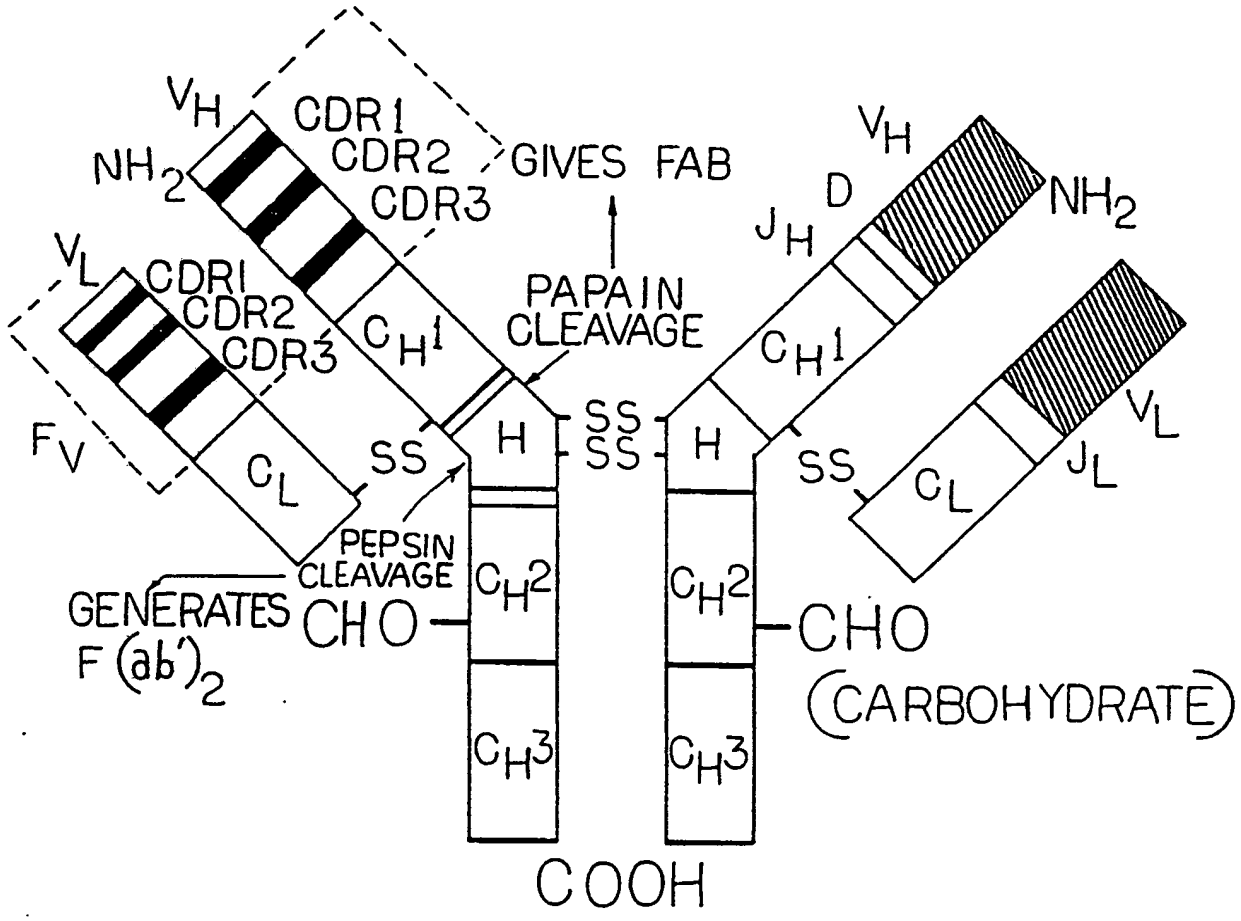

V_H
CDR1
CDR2
CDR3
NH_2
GIVES FAB
V_L
CDR1
CDR2
CDR3
C_H1
PAPAIN CLEAVAGE
F_V
C_L
SS
H
SS
SS
H
V_H
D
J_H
NH_2
C_H1
SS
C_L
J_L
V_L
PEPSIN CLEAVAGE
GENERATES $F(ab')_2$
CHO
C_H2
C_H2
CHO
(CARBOHYDRATE)
C_H3
C_H3
COOH

FIG.2

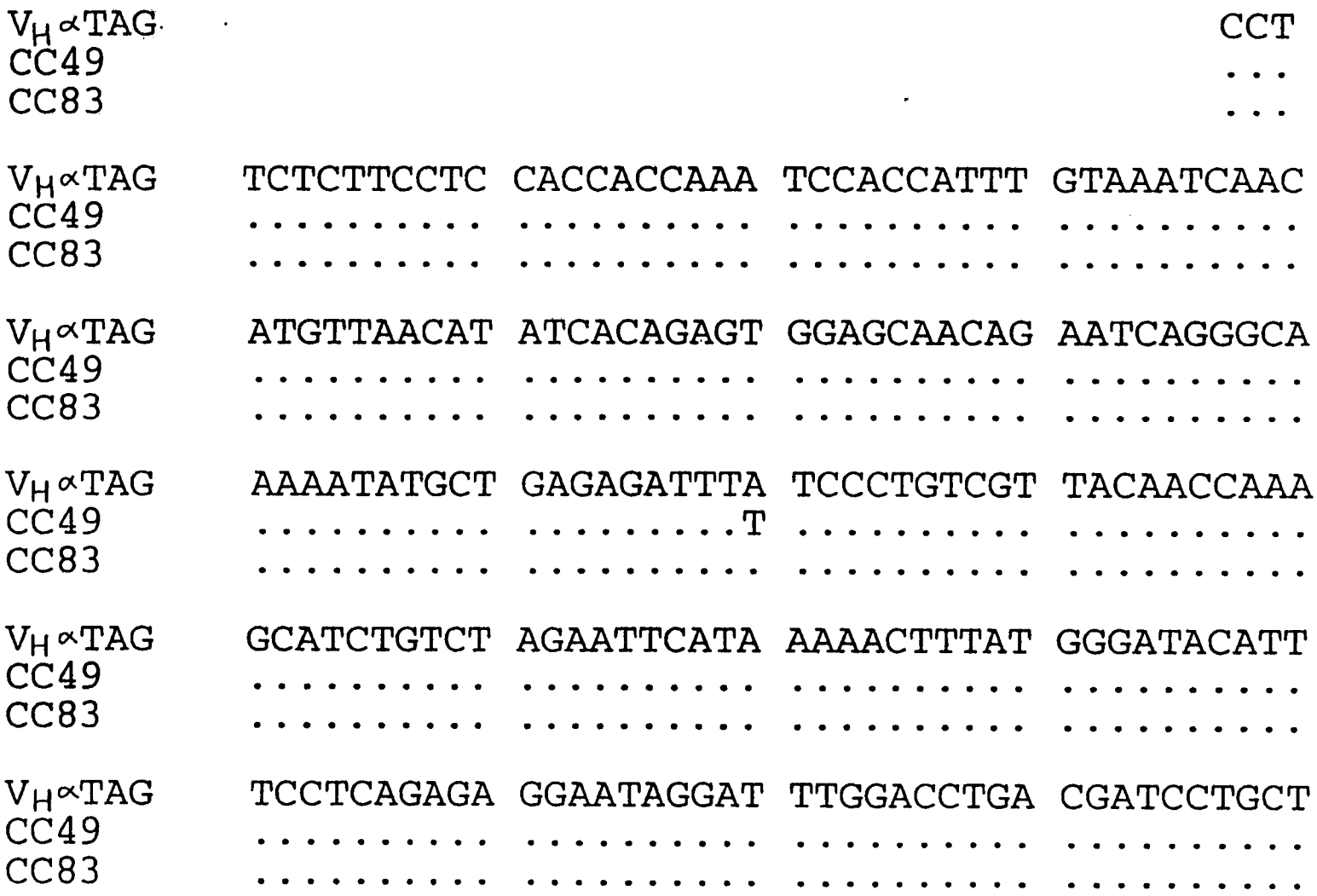

$V_H\alpha$TAG CCT
CC49 ...
CC83 ...
$V_H\alpha$TAG TCTCTTCCTC CACCACCAAA TCCACCATTT GTAAATCAAC
CC49 ....
CC83 ....
$V_H\alpha$TAG ATGTTAACAT ATCACAGAGT GGAGCAACAG AATCAGGGCA
CC49 ....
CC83 ....
$V_H\alpha$TAG AAAATATGCT GAGAGATTTA TCCCTGTCGT TACAACCAAA
CC49 ....T ....
CC83 ....
$V_H\alpha$TAG GCATCTGTCT AGAATTCATA AAAACTTTAT GGGATACATT
CC49 ....
CC83 ....
$V_H\alpha$TAG TCCTCAGAGA GGAATAGGAT TTGGACCTGA CGATCCTGCT
CC49 ....
CC83 ....

FIG. 2 (CONT.)

$V_H\alpha$TAG	GCCCGAGCCA	TGTGATGACA	GTTCTTCTCC	AGTTGAACTA
CC49	....	....	....	....
CC83	....	....	....	....
$V_H\alpha$TAG	GGTCCTTATC	TAAGAAATGC	ACTGCTCATG	AATATGCAAA
CC49	....	....	....	....
CC83	....	....	....	....
$V_H\alpha$TAG	TCACCCGAGT	CTATGGCAGT	AAATACAGAG	ATGTTCATAC
CC49	....	....	....	....
CC83	....	....	....	....
$V_H\alpha$TAG	CATAAAAACA	ATATATGATC	AGTGTCTTCT	CCGCTATCCC
CC46		....	....	....
CC49	....	....G.....	....	....
CC83	....	....	....	....
CC92				....

FIG. 2 (CONT.)

V_H∝TAG	TGGACACACT	GACTCTAACC	ATG	GAA	TGG	AGC	TGG			
CC46	....	....	...	...	...	...	...			
CC49	....	....	...	...	...	...	...			
CC83	....	....	...	...	...	...	...			
CC92	....	....	...	...	...	...	...			

V_H∝TAG	GTC	TTT	CTC	TTC	TTC	CTG	TCA	GTA	ACT	ACA	G
CC46	...	...	...	...	...	...	...	...	...	...	.
CC49	...	...	...	...	...	...	...	...	...	...	.
CC83	...	...	...	...	...	...	...	...	...	...	.
CC92	...	...	...	...	...	...	...	...	...	...	.

V_H∝TAG	GTAAGGGGCT	CACCATTTCC	AAATCTAAAG	TGGAGTCAGG
CC46				
CC49	....	....	....	....
CC83	....	....	....	....
CC92				

FIG. 2 (CONT.)

```
VHαTAG   GCCTGAGGTG ACAAAGATAT CACCTTTGGC TTTCCACAG
CC46
CC49     ......... ........G. ......... .G.......
CC83     ......... ......... ......... .........
CC92

VHαTAG   GT GTC CAC TCC CAG GTT CAG CTG CAG CAG TCT
CC46     .. ... ... ... ..A T.. ... ..A ... ... ...
CC49     .. ... ... ... ... ... ... T.. ... ... ...
CC83     .. ... ... ... ... ... ... T.. ... ... ...
CC92     .. ... ... ... ... ... ..A ... ... ... ...

VHαTAG   GAC GCT GAG TTG GTG AAA CCT GGG GCT TCA GTG
CC46     ... ... ... ... ... .G. ... ... ... ... ...
CC49     ... ... ... ... ... ... ... ... ... ... ...
CC83     ... ... ... ... ... ... ... ... ... ... ...
CC92     ... ... ... ... ... ... ... ... ... ... ...
```

FIG. 2 (CONT.)

$V_H\alpha$TAG	AAG	ATA	TCC	TGC	AAG	GCT	TCT	GGC	TAC	ACC	TTC
CC46	...	..T	...	...	...	...	...	...	...	...	...
CC49	...	..T	...	...	...	...	...	...	...	...	...
CC83	...	..T	...	...	...	...	...	...	...	...	...
CC92	...	...	...	...	...	...	...	...	...	...	...

CDR1

$V_H\alpha$TAG	ACT	GAC	CAT	GCT	ATT	CAC	TGG	GTG	AAG	CAG	AAG
CC46	...	...	...	...	...	...	...	...	...	...	...
CC49	...	...	...	..A	...	...	...	...	..A	...	..C
CC83	...	...	...	...	...	...	...	...	...	...	...
CC92	...	...	...	...	...	...	...	...	...	...	..A

$V_H\alpha$TAG	CCT	GAA	CAG	GGC	CTG	GAA	TGG	ATT	GGA	TAT	ATT
CC46	...	...	...	...	...	...	...	...	...	...	T..
CC49	...	...	...	...	...	...	...	...	...	...	T..
CC83	...	...	...	...	...	...	...	...	...	...	...
CC92	...	...	...	...	...	...	...	...	...	...	...

CDR2

$V_H\alpha$TAG	TCT	CCC	GGA	AAT	GGT	GAT	ATT	AAG	TAC	AAT	GAG
CC46	...	...	...	...	...	...	...	...	...	...	...
CC49	...	...	...	...	.A.	...	T..	..A	...	...	...
CC83	...	...	...	...	.A.	...	...	...	...	...	...
CC92	...	...	...	...	.A.	...	...	...	...	...	...

FIG. 2 (CONT.)

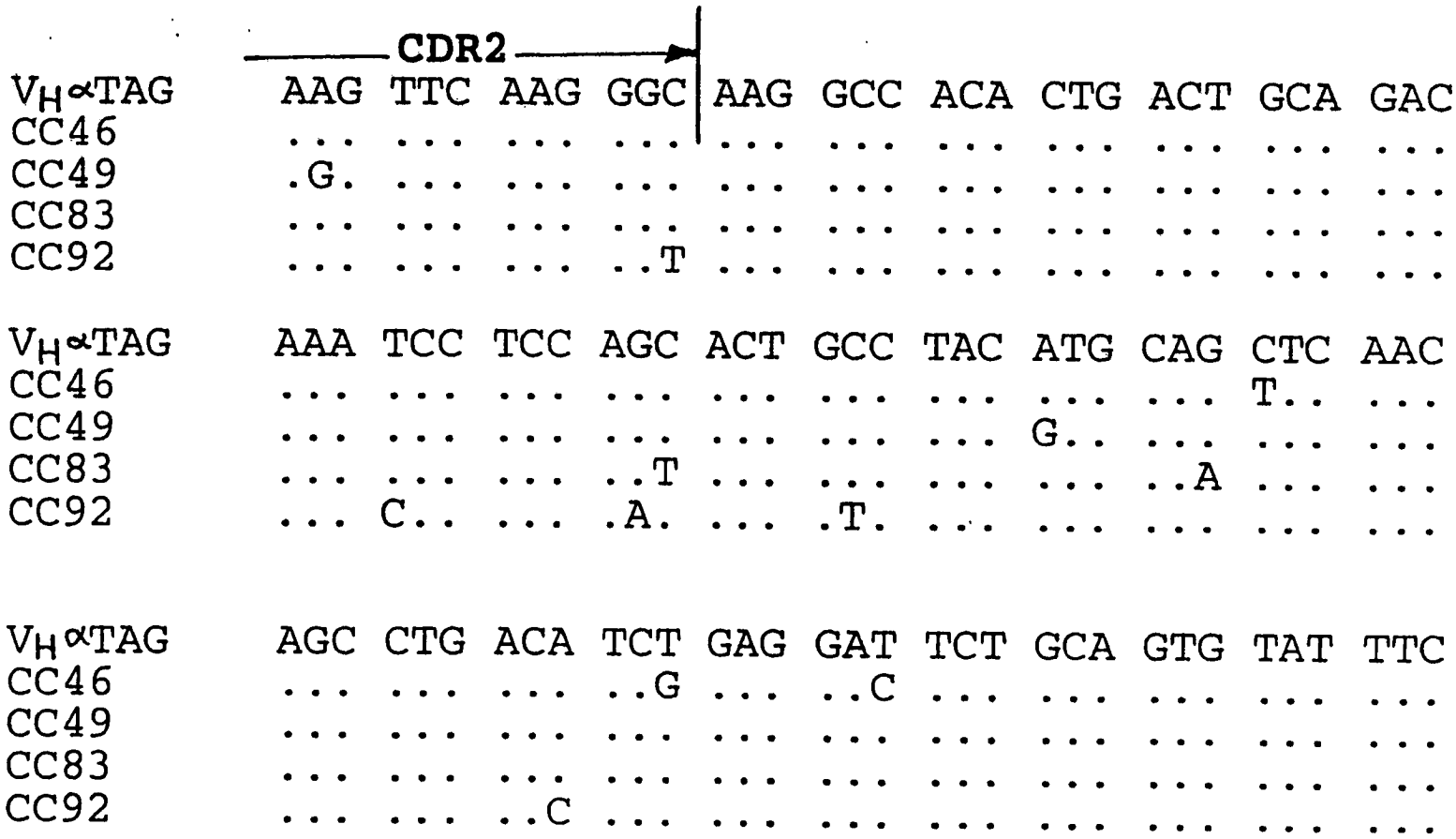

CDR2
VH∝TAG AAG TTC AAG GGC AAG GCC ACA CTG ACT GCA GAC
CC46 ... ...
CC49 .G. ... ...
CC83 ... ...
CC92 ... ...T ... ...
VH∝TAG AAA TCC TCC AGC ACT GCC TAC ATG CAG CTC AAC
CC46 ... ... T.. ...
CC49 ... ... G.. ... ...
CC83 ... ...T ... ...A ... ...
CC92 ... C.. ... .A. ... .T. ... ...
VH∝TAG AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT TTC
CC46 ... ...G ... ..C ... ...
CC49 ... ...
CC83 ... ...
CC92 ... .C ... ...

FIG. 2 (CONT.)

V_H∝TAG	TGT	AAA	AGA	CACAGTGTTG	TAACCACATC	CTGAGTGTGT				
CC46	...	.CG	G.C	GGC	TAC	GGG	GTT	GCT	TTC	TGG	GGC
CC49	...	.C.	...	TCC	CTG	AAT	ATG	GCC	TAC	TGG	GGT
CC83	...	.G.	...	TCC	TTC	TAC	GGC	AAC	---	TGG	GGC
CC92	...	.C.	...	TCT	CTA	TCC	GGG	GAC	TCC	TGG	GGC

CDR3

V_H∝TAG	CAGAAATCCT	GGGGGAGCAG	AAAGATACAC	TGGGACTGAG					
CC46	CAA	GGG	ACT	CTG	GTC	ACT	GTC	TCT	GCA	G
CC49	CAA	GGA	ACC	TCA	GTC	ACC	GTC	TCC	TCA	G
CC83	CAA	GGC	ACC	ACC	CTC	ACA	GTC	TCC	TCA	G
CC92	CAG	GGC	ACC	ACT	CTC	ACA	GTC	TCC	TCA	G

V_H∝TAG AAGACAGAAA AATTAATCCT TAGACTTGCT CAGAAATCGT

V_H∝TAG AATTTTGAAT GCCTATTTAT TTCATCTTGC TCACACACCT

V_H∝TAG ATATTGCTTT TGTAAGCTT

FIG. 2 (CONT.)

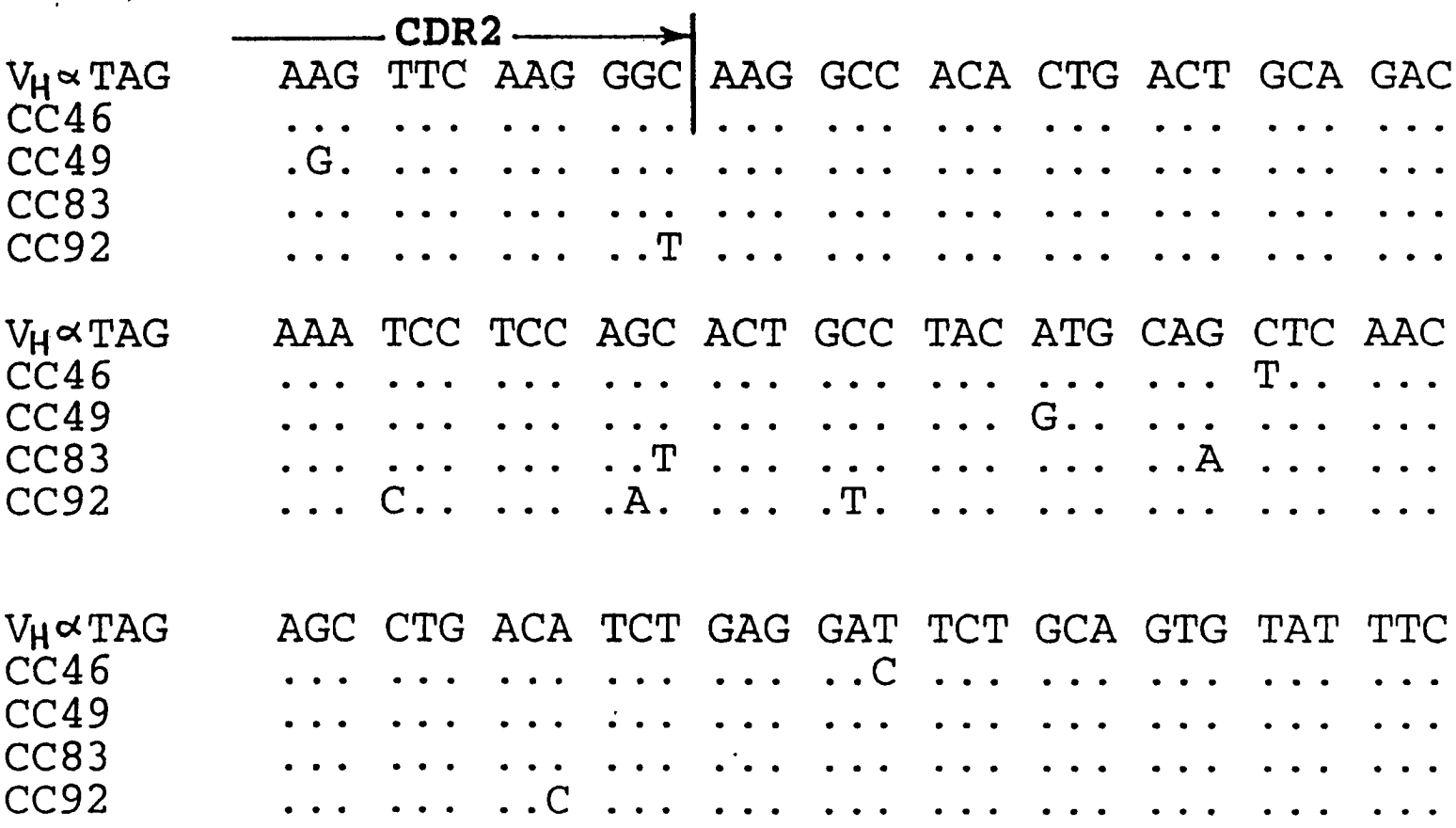

CDR2
VH∝TAG AAG TTC AAG GGC AAG GCC ACA CTG ACT GCA GAC
CC46 ... ...
CC49 .G. ... ...
CC83 ... ...
CC92 ... ...T ... ...
VH∝TAG AAA TCC TCC AGC ACT GCC TAC ATG CAG CTC AAC
CC46 ... ... T.. ...
CC49 ... ... G.. ... ...
CC83 ... ...T ... ...A ... ...
CC92 ... C.. ... .A. ... .T. ... ...
VH∝TAG AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT TTC
CC46 ... ...C ... ...
CC49 ... ...
CC83 ... ...
CC92 ... .C ... ...

FIG. 2 (CONT.)

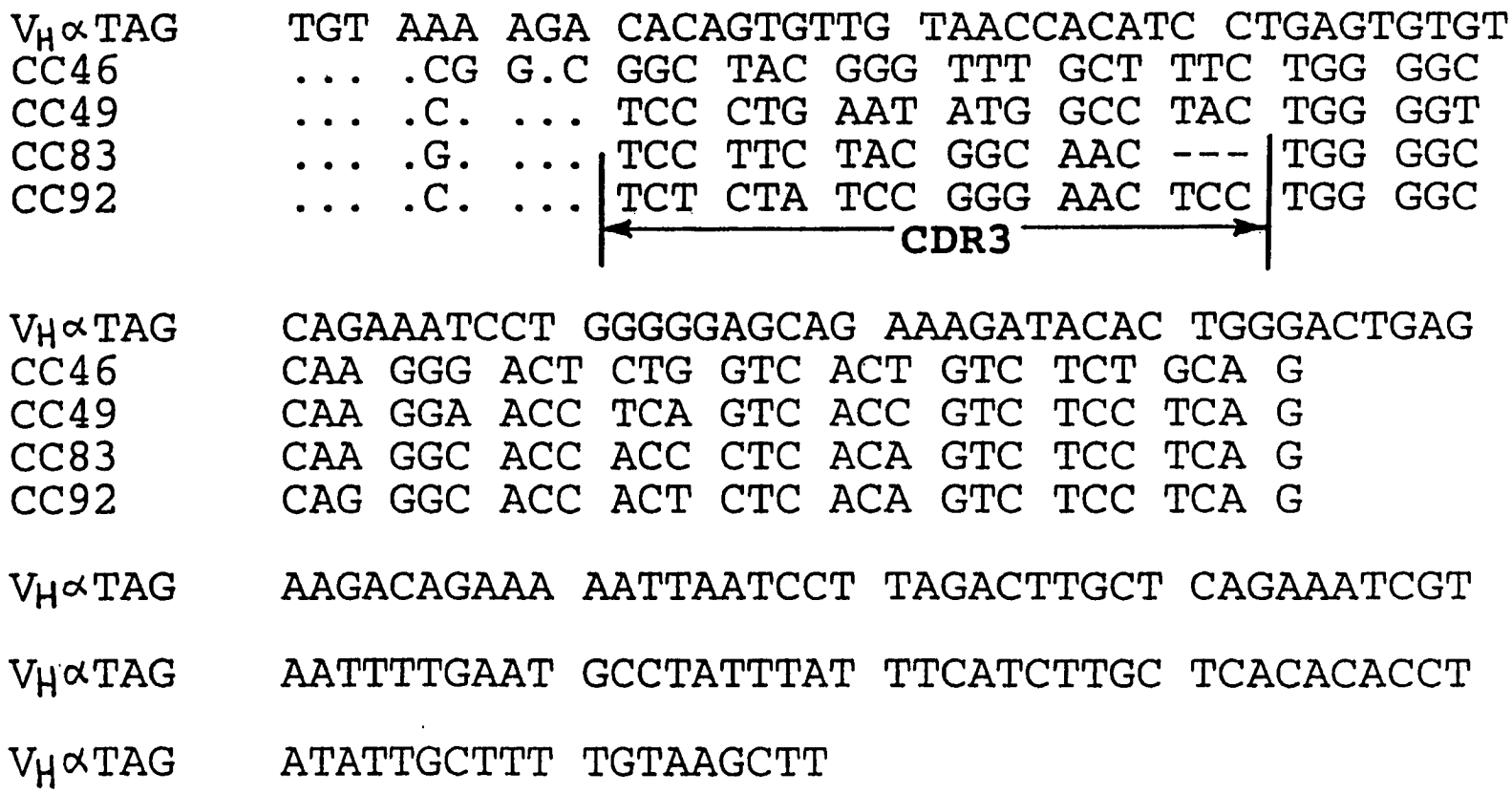

VHαTAG TGT AAA AGA CACAGTGTTG TAACCACATC CTGAGTGTGT
CC46 ... .CG G.C GGC TAC GGG TTT GCT TTC TGG GGC
CC49 ... .C. ... TCC CTG AAT ATG GCC TAC TGG GGT
CC83 ... .G. ... TCC TTC TAC GGC AAC --- TGG GGC
CC92 ... .C. ... TCT CTA TCC GGG AAC TCC TGG GGC
CDR3
VHαTAG CAGAAATCCT GGGGGAGCAG AAAGATACAC TGGGACTGAG
CC46 CAA GGG ACT CTG GTC ACT GTC TCT GCA G
CC49 CAA GGA ACC TCA GTC ACC GTC TCC TCA G
CC83 CAA GGC ACC ACC CTC ACA GTC TCC TCA G
CC92 CAG GGC ACC ACT CTC ACA GTC TCC TCA G
VHαTAG AAGACAGAAA AATTAATCCT TAGACTTGCT CAGAAATCGT
VHαTAG AATTTTGAAT GCCTATTTAT TTCATCTTGC TCACACACCT
VHαTAG ATATTGCTTT TGTAAGCTT

FIG.3

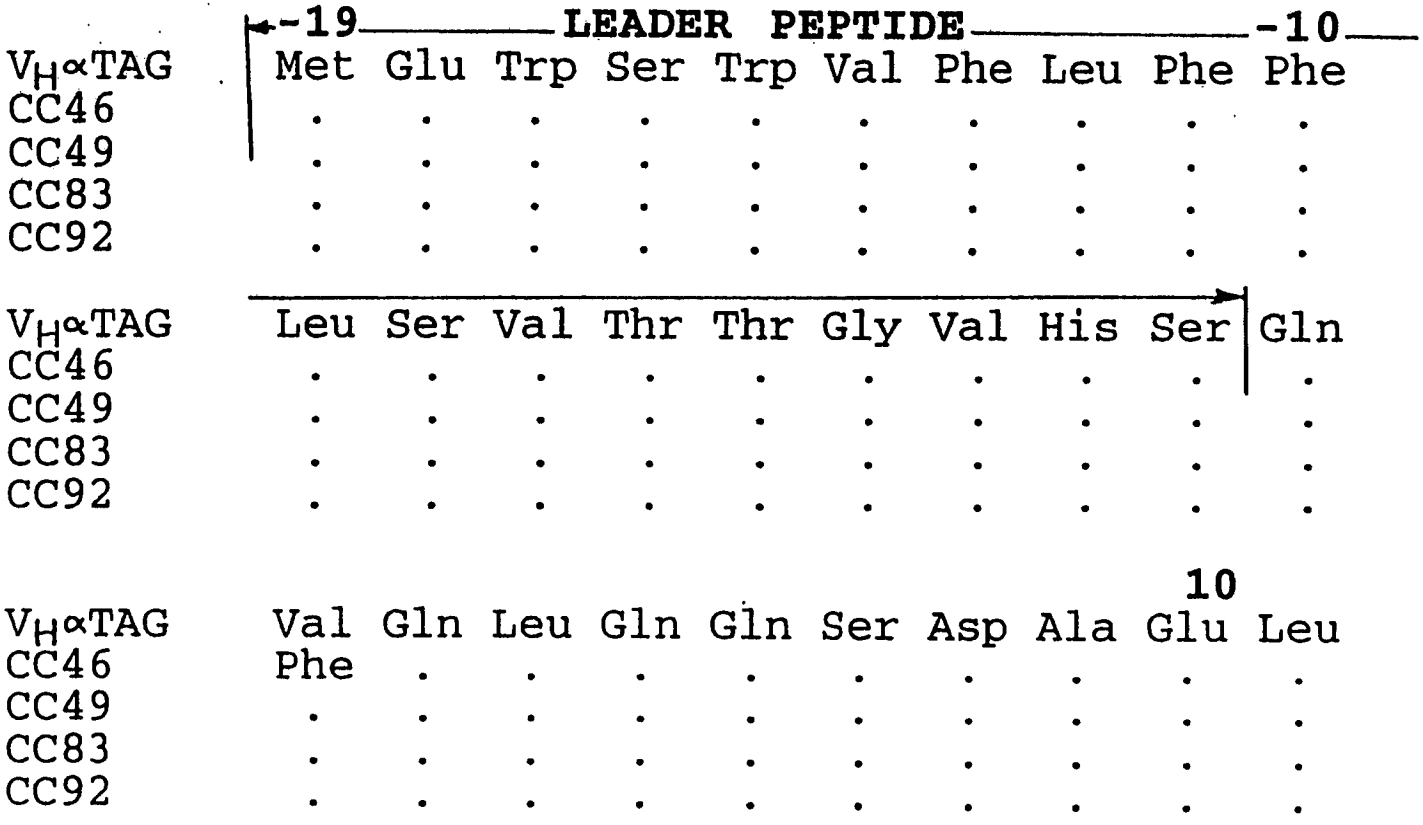

-19
LEADER PEPTIDE
-10
VHαTAG Met Glu Trp Ser Trp Val Phe Leu Phe Phe
CC46 . . . .
CC49 . . . .
CC83 . . . .
CC92 . . . .
VHαTAG Leu Ser Val Thr Thr Gly Val His Ser Gln
CC46 . . . .
CC49 . . . .
CC83 . . . .
CC92 . . . .
10
VHαTAG Val Gln Leu Gln Gln Ser Asp Ala Glu Leu
CC46 Phe . . . .
CC49 . . . .
CC83 . . . .
CC92 . . . .

FIG. 3 (CONT.)

									20	
$V_H\alpha$TAG	Val	Lys	Pro	Gly	Ala	Ser	Val	Lys	Ile	Ser
CC46	.	Arg	.	.	.	.	.	.	.	.
CC49	.	.	.	.	.	.	.	.	.	.
CC83	.	.	.	.	.	.	.	.	.	.
CC92	.	.	.	.	.	.	.	.	.	.

								30	←	
$V_H\alpha$TAG	Cys	Lys	Ala	Ser	Gly	Tyr	Thr	Phe	Thr	Asp
CC46	.	.	.	.	.	.	.	.	.	.
CC49	.	.	.	.	.	.	.	.	.	.
CC83	.	.	.	.	.	.	.	.	.	.
CC92	.	.	.	.	.	.	.	.	.	.

	←	CDR1	→					40	
$V_H\alpha$TAG	His	Ala	Ile	His	Trp	Val	Lys	Gln	Lys	Pro
CC46	.	.	.	.	.	.	.	.	.	.
CC49	.	.	.	.	.	.	.	.	Asn	.
CC83	.	.	.	.	.	.	.	.	.	.
CC92	.	.	.	.	.	.	.	.	.	.

									← 50	
$V_H\alpha$TAG	Glu	Gln	Gly	Leu	Glu	Trp	Ile	Gly	Tyr	Ile
CC46	.	.	.	.	.	.	.	.	.	Phe
CC49	.	.	.	.	.	.	.	.	.	Phe
CC83	.	.	.	.	.	.	.	.	.	.
CC92	.	.	.	.	.	.	.	.	.	.

FIG. 3 (CONT.)

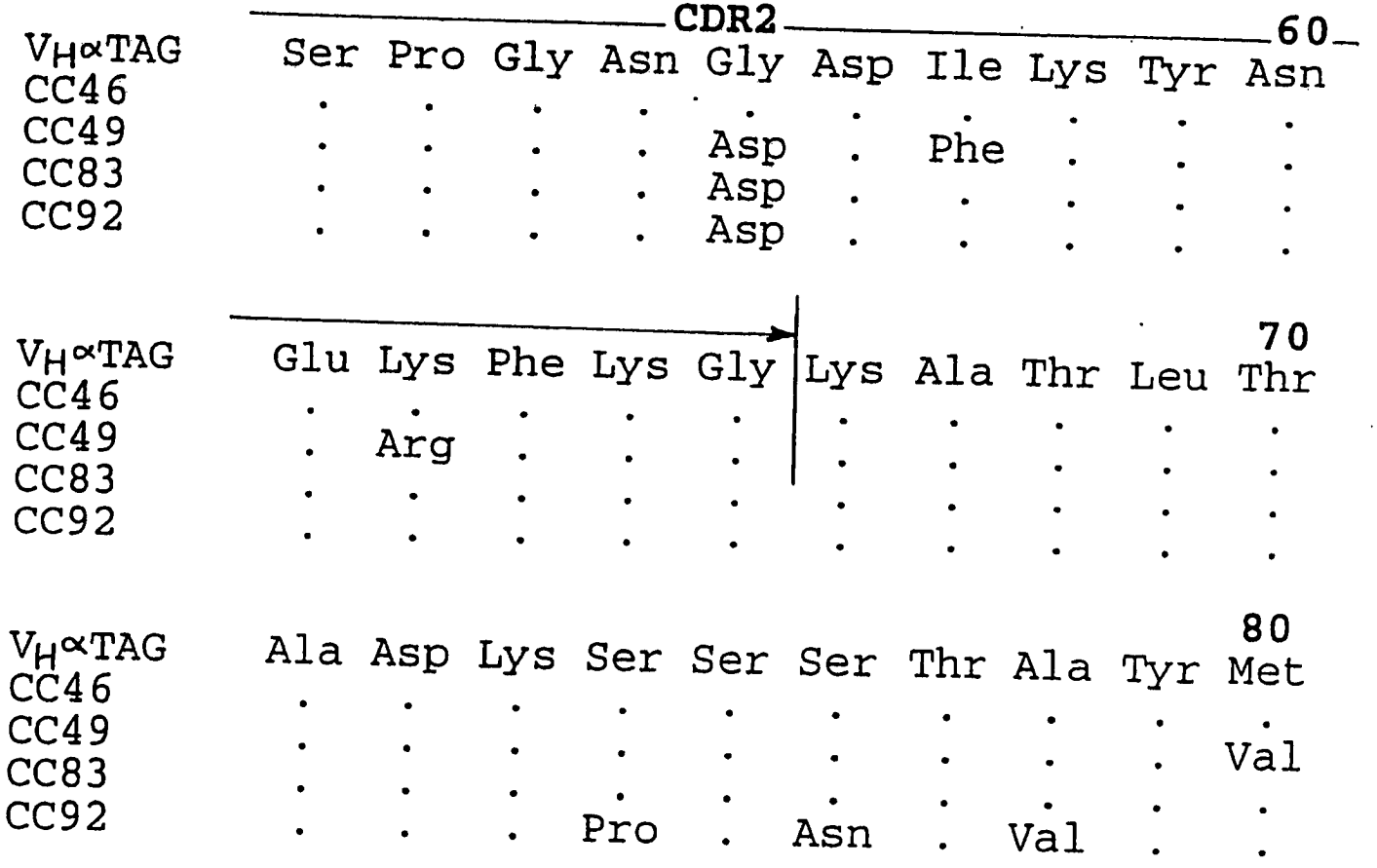

CDR2
60
VH∝TAG Ser Pro Gly Asn Gly Asp Ile Lys Tyr Asn
CC46
CC49 Asp Phe
CC83 Asp
CC92 Asp
70
VH∝TAG Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr
CC46
CC49 Arg
CC83
CC92
80
VH∝TAG Ala Asp Lys Ser Ser Ser Thr Ala Tyr Met
CC46
CC49 Val
CC83
CC92 Pro Asn Val

FIG. 3 (CONT.)

	110
CC46	Gly Thr Leu Val Thr Val Ser Ala
CC49	Gly Thr Ser Val Thr Val Ser Ser
CC83	Gly Thr Thr Leu Thr Val Ser Ser
CC92	Gly Thr Thr Leu Thr Val Ser Ser

FIG. 3 (CONT.)

$V_H\alpha$TAG	Gln	Leu	Asn	Ser	Leu	Thr	Ser	Glu	Asp	Ser
CC46	.	Phe	.	.	.	.	.	.	.	.
CC49	.	.	.	.	.	.	.	.	.	.
CC83	.	.	.	.	.	.	.	.	.	.
CC92										

			90					
$V_H\alpha$TAG	Ala	Val	Tyr	Phe	Cys	Lys	Arg	
CC46	.	.	.	.	.	Thr	Gly	
CC49	.	.	.	.	.	Thr	.	
CC83	.	.	.	.	.	Arg	.	
CC92	.	.	.	.	.	Thr	.	

	←——— CDR3 ———→							105
CC46	Gly	Tyr	Gly	Val	Ala	Phe	Trp	Gly	Gln
CC49	Ser	Leu	Asn	Met	Ala	Tyr	Trp	Gly	Gln
CC83	Ser	Phe	Tyr	Gly	Asn	-	Trp	Gly	Gln
CC92	Ser	Leu	Ser	Gly	Asp	Ser	Trp	Gly	Gln

FIG. 3 (CONT.)

V_HαTAG	Gln	Leu	Asn	Ser	Leu	Thr	Ser	Glu	Asp	Ser
CC46	.	Phe	.	.	.	.	.	.	.	.
CC49	.	.	.	.	.	.	.	.	.	.
CC83	.	.	.	.	.	.	.	.	.	.
CC92										

			90				
V_HαTAG	Ala	Val	Tyr	Phe	Cys	Lys	Arg
CC46	.	.	.	.	.	Thr	Gly
CC49	.	.	.	.	.	Thr	.
CC83	.	.	.	.	.	Arg	.
CC92	.	.	.	.	.	Thr	.

	CDR3								105
CC46	Gly	Tyr	Gly	Phe	Ala	Phe	Trp	Gly	Gln
CC49	Ser	Leu	Asn	Met	Ala	Tyr	Trp	Gly	Gln
CC83	Ser	Phe	Tyr	Gly	Asn	-	Trp	Gly	Gln
CC92	Ser	Leu	Ser	Gly	Asn	Ser	Trp	Gly	Gln

FIG. 4

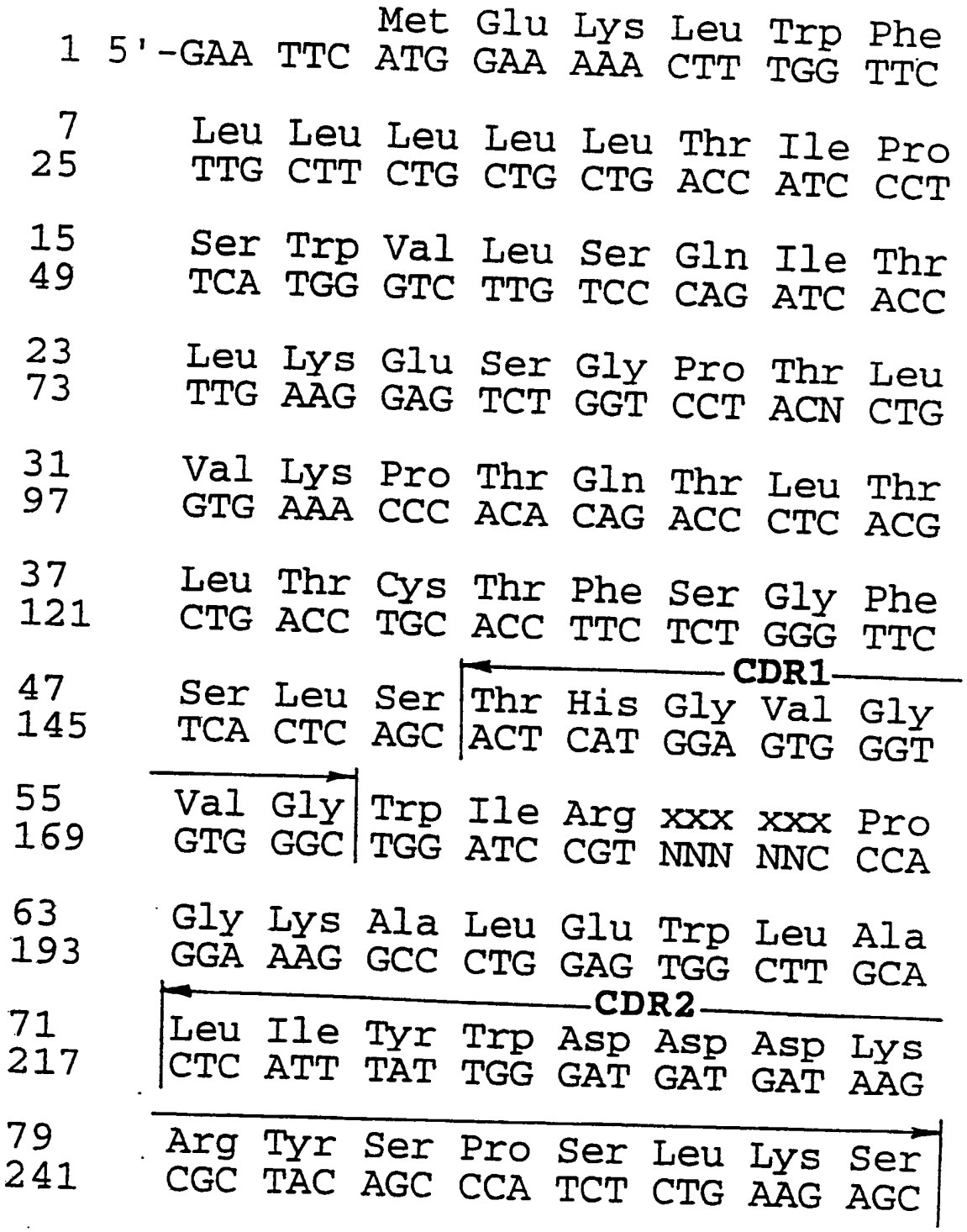

Met Glu Lys Leu Trp Phe
1 5'-GAA TTC ATG GAA AAA CTT TGG TTC
7 Leu Leu Leu Leu Leu Thr Ile Pro
25 TTG CTT CTG CTG CTG ACC ATC CCT
15 Ser Trp Val Leu Ser Gln Ile Thr
49 TCA TGG GTC TTG TCC CAG ATC ACC
23 Leu Lys Glu Ser Gly Pro Thr Leu
73 TTG AAG GAG TCT GGT CCT ACN CTG
31 Val Lys Pro Thr Gln Thr Leu Thr
97 GTG AAA CCC ACA CAG ACC CTC ACG
37 Leu Thr Cys Thr Phe Ser Gly Phe
121 CTG ACC TGC ACC TTC TCT GGG TTC
CDR1
47 Ser Leu Ser Thr His Gly Val Gly
145 TCA CTC AGC ACT CAT GGA GTG GGT
55 Val Gly Trp Ile Arg xxx xxx Pro
169 GTG GGC TGG ATC CGT NNN NNC CCA
63 Gly Lys Ala Leu Glu Trp Leu Ala
193 GGA AAG GCC CTG GAG TGG CTT GCA
CDR2
71 Leu Ile Tyr Trp Asp Asp Asp Lys
217 CTC ATT TAT TGG GAT GAT GAT AAG
79 Arg Tyr Ser Pro Ser Leu Lys Ser
241 CGC TAC AGC CCA TCT CTG AAG AGC

FIG. 4 (CONT.)

87	Arg	Leu	Thr	Ile	Thr	Lys	Asp	Thr
265	AGG	CTC	ACC	ATC	ACC	AAG	GAC	ACC
95	Ser	Lys	Asn	Gln	Val	Ile	Leu	Thr
289	TCC	AAA	AAC	CAG	GTG	ATC	CTT	ACA
103	Met	Thr	Asn	Met	Asp	Pro	Val	Asp
313	ATG	ACC	AAC	ATG	GAC	CCT	GTG	GAC
111	Thr	Ala	Thr	Tyr	Tyr	Cys	Ala	His
337	ACA	GCC	ACA	TAT	TAT	TGT	GCA	CAC
	←			**CDR3**				→
119	Gly	Leu	Pro	Ser	Met	Val	Lys	Asn
361	GGG	CTG	CCA	TCT	ATG	GTT	AAG	AAC
127	Trp	Gly	Gln	Gly	Thr	Thr	Val	Thr
385	TGG	GGC	CAA	GGG	ACC	ACG	GTC	ACC
135	Val	Ser	Ser	Gly	Ser			
409	GTC	TCC	TCA	GGG	AGT-3'			

FIG. 4

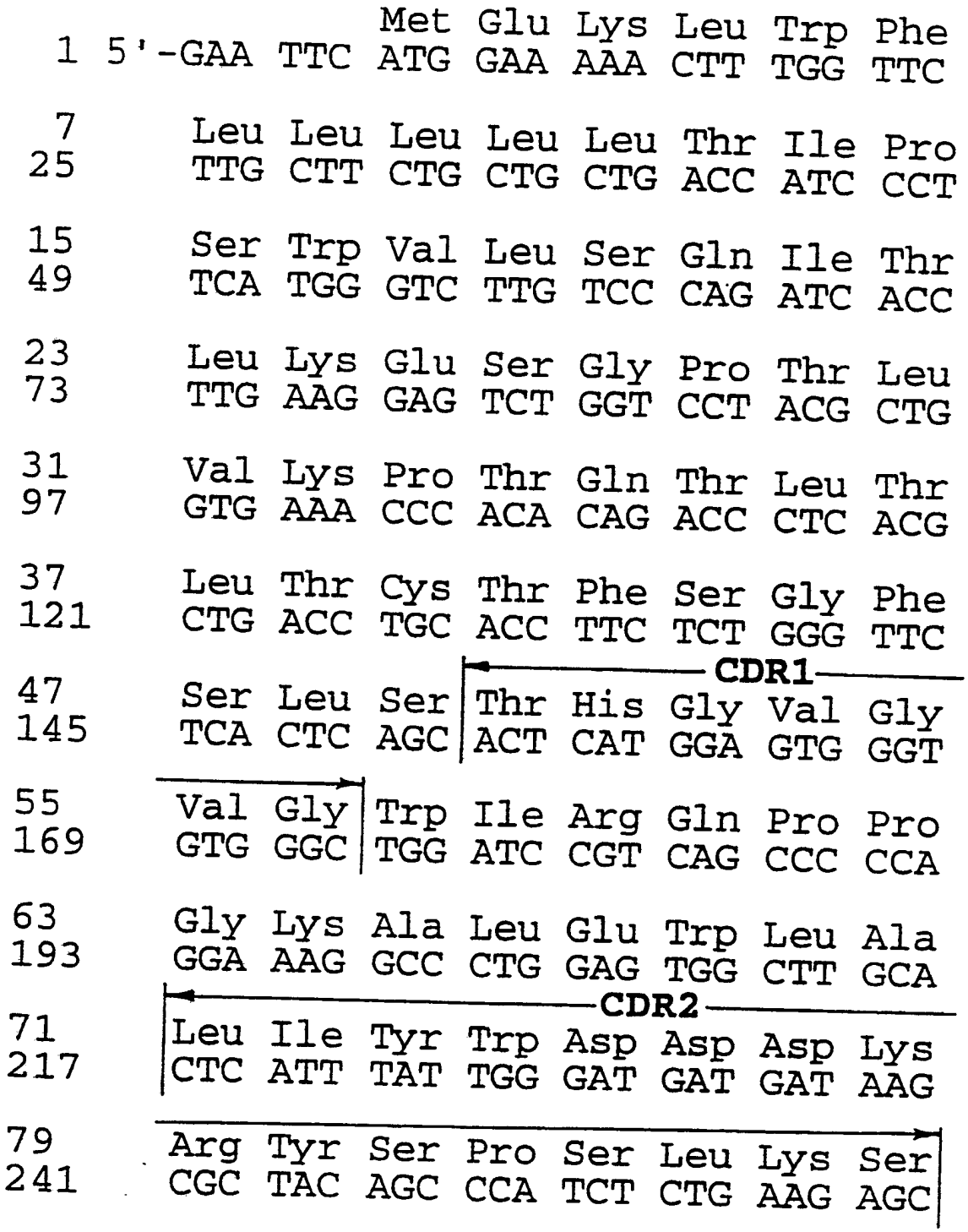

Met Glu Lys Leu Trp Phe
1 5'-GAA TTC ATG GAA AAA CTT TGG TTC
7 Leu Leu Leu Leu Leu Thr Ile Pro
25 TTG CTT CTG CTG CTG ACC ATC CCT
15 Ser Trp Val Leu Ser Gln Ile Thr
49 TCA TGG GTC TTG TCC CAG ATC ACC
23 Leu Lys Glu Ser Gly Pro Thr Leu
73 TTG AAG GAG TCT GGT CCT ACG CTG
31 Val Lys Pro Thr Gln Thr Leu Thr
97 GTG AAA CCC ACA CAG ACC CTC ACG
37 Leu Thr Cys Thr Phe Ser Gly Phe
121 CTG ACC TGC ACC TTC TCT GGG TTC
CDR1
47 Ser Leu Ser Thr His Gly Val Gly
145 TCA CTC AGC ACT CAT GGA GTG GGT
55 Val Gly Trp Ile Arg Gln Pro Pro
169 GTG GGC TGG ATC CGT CAG CCC CCA
63 Gly Lys Ala Leu Glu Trp Leu Ala
193 GGA AAG GCC CTG GAG TGG CTT GCA
CDR2
71 Leu Ile Tyr Trp Asp Asp Asp Lys
217 CTC ATT TAT TGG GAT GAT GAT AAG
79 Arg Tyr Ser Pro Ser Leu Lys Ser
241 CGC TAC AGC CCA TCT CTG AAG AGC

FIG. 5

MOUSE GERMLINE J-H GENES FROM pNP9

5'-GGATCCTGGC CAGCATTGCC GCTAGGTCCC
TCTCTTCTAT GCTTTCTTTG TCCCTCACTG
GCCTCCATCT GAGATAATCC TGGAGCCCTA
GCCAAGGATC ATTTATTGTC AGGGGTCTAA
TCATTGTTGT CACAATGTGC CTGGTTTGCT
TACTGGGGCC AAGGGACTCT GGTCACTGTC
TCTGCAGGTG AGTCCTAACT TCTCCCATTC
TAAATGCATG TTGGGGGGAT TCTGAGCCTT
CAGGACCAAG ATTCTCTGCA AACGGGAATC
AAGATTCAAC CCCTTTGTCC CAAAGTTGAG
ACATGGGTCT GGGTCAGGGA CTCTCTGCCT
GCTGGTCTGT GGTGACATTA GAACTGAAGT
ATGATGAAGG ATCTGCCAGA ACTGAAGCTT
GAAGTCTGAG GCAGAATCTT GTCCAGGGTC
TATCGGACTC TTGTGAGAAT TAGGGGCTGA
CAGTTGATGG TGACAATTTC AGGGTCAGTG
ACTGTCAGGT TTCTCTGAGG TGAGGCTGGA
ATATAGGTCA CCTTGAAGAC TAAAGAGGGG
TCCAGGGGCT TTTCTGCACA GGCAGGGAAC
AGAATGTGGA ACAATGACTT GAATGGTTGA
TTCTTGTGTG ACACCAAGAA TTGGCATAAT
GTCTGAGTTG CCCAAGGGTG ATCTTAGCTA
AAAACCCACT ATTGTGATTA CTATGCTATG
GACTACTGGG GTCAAGGAAC CTCAGTCACC
GTCTCCTCAG GTAAGAATGG CCTCTCCAGG
TCTTTATTTT TAACCTTTGT TATGGAGTTT
TCTGAGCATT GCAGACTAAT CTTGGATATT
TGCCCTGAGG GAGCCGGCTG AGAGAAGTTG
GGAAATAAAT CTGTCTAGGG ATCTCAGAGC
CTTTAGGACA GATTATCTCC ACATCTTTGA
AAAACTAAGA ATCTGTGTGA TGGTGTTGGT
GGAGTCCCTG GATGATGGGA TAGGGACTTT

FIG. 5 (CONT.)

GGAGGCTCAT TTGAGGGAGA TGCTAAAACA
ATCCTATGGC TGGAGGGATA GTTGGGGCTG
TAGTTGGAGA TTTTCAGTTT TTAGAATGAA
GTATTAGCTG CAATACTTCA AGGACCACCT
CTGTGACAAC CATTTTATAC AGTATCCAGG
CATAGGGACA AAAAGTGGAG TGGGGCACTT
TCTTTAGATT TGTGAGGAAT GTTCCACACT
AGATTGTTTA AAACTTCATT TGTTGGAAGG
AGCTGTCTTA GTGATTGAGT CAAGGGAGAA
AGGCATCTAG CCTCGGTCTC AAAAGGGTAG
TTGCTGTCTA GAGAGGTCTG GTGGAGCCTG
CAAAAGTCCA GCTTTCAAAG GAACACAGAA
GTATGTGTAT GGAATATTAG AAGATGTTGC
TTTTACTCTT AAGTTGGTTC CTAGGAAAAA
TAGTTAAATA CTGTGACTTT AAAATGTGAG
AGGGTTTTCA AGTACTCATT TTTTTAAATG
TCCAAAATTT TTGTCAATCA ATTTGAGGTC
TTGTTTGTGT AGAACTGACA TTACTTAAAG
TTTAACCGAG GAATGGGAGT GAGGCTCTCT
CATACCCTAT TCAGAACTGA CTTTTAACAA
TAATAAATTA AGTTTAAAAT ATTTTTAAAT
GAATTGAGCA ATGTTGAGTT GAGTCAAGAT
GGCCGATCAG AACCGGAACA CCTGCAGCAG
CTGGCAGGAA GCAGGTCATG TGGCAAGGCT
ATTTGGGGAA GGGAAAATAA AACCACTAGG
TAAACTTGTA GCTGTGGTTT GAAGAAGTGG
TTTTGAAACA CTCTGTCCAG CCCCACCAAA
CCGAAAGTCC AGGCTGAGCA AAACACCACC
TGGGTAATTT GCATTTCTAA AATAAGTTGA
GGATTCAGCC GAAACTGGAG AGGTCCTCTT
TTAACTTATT GAGTTCAACC TTTTAATTTT
AGCTTGAGTA GTTCTAGTTT CCCCAAACTT
AAGTTTATCG ACTTCTAAAA TGTATTTAGA
ATTC-3'

Fig. 6

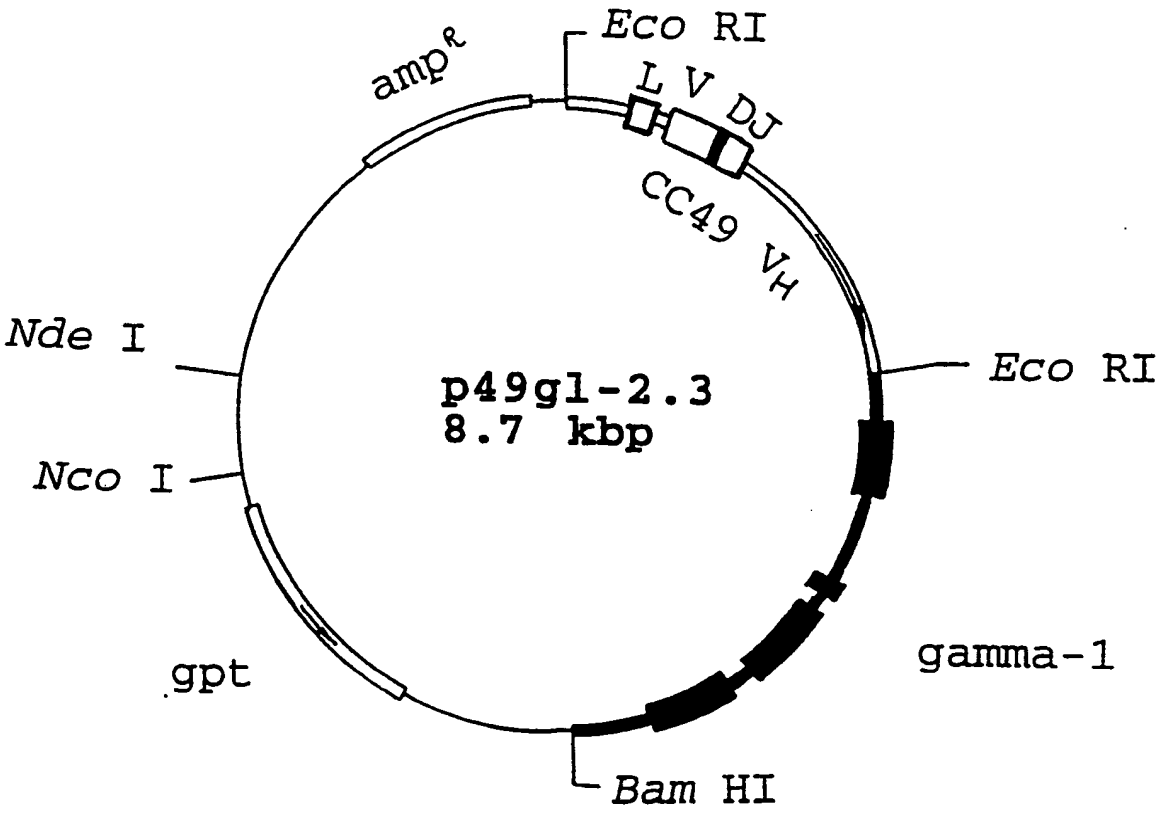

amp^R
Eco RI
L V DJ
CC49 VH
Nde I
p49g1-2.3
8.7 kbp
Eco RI
Nco I
gamma-1
gpt
Bam HI

FIG. 7

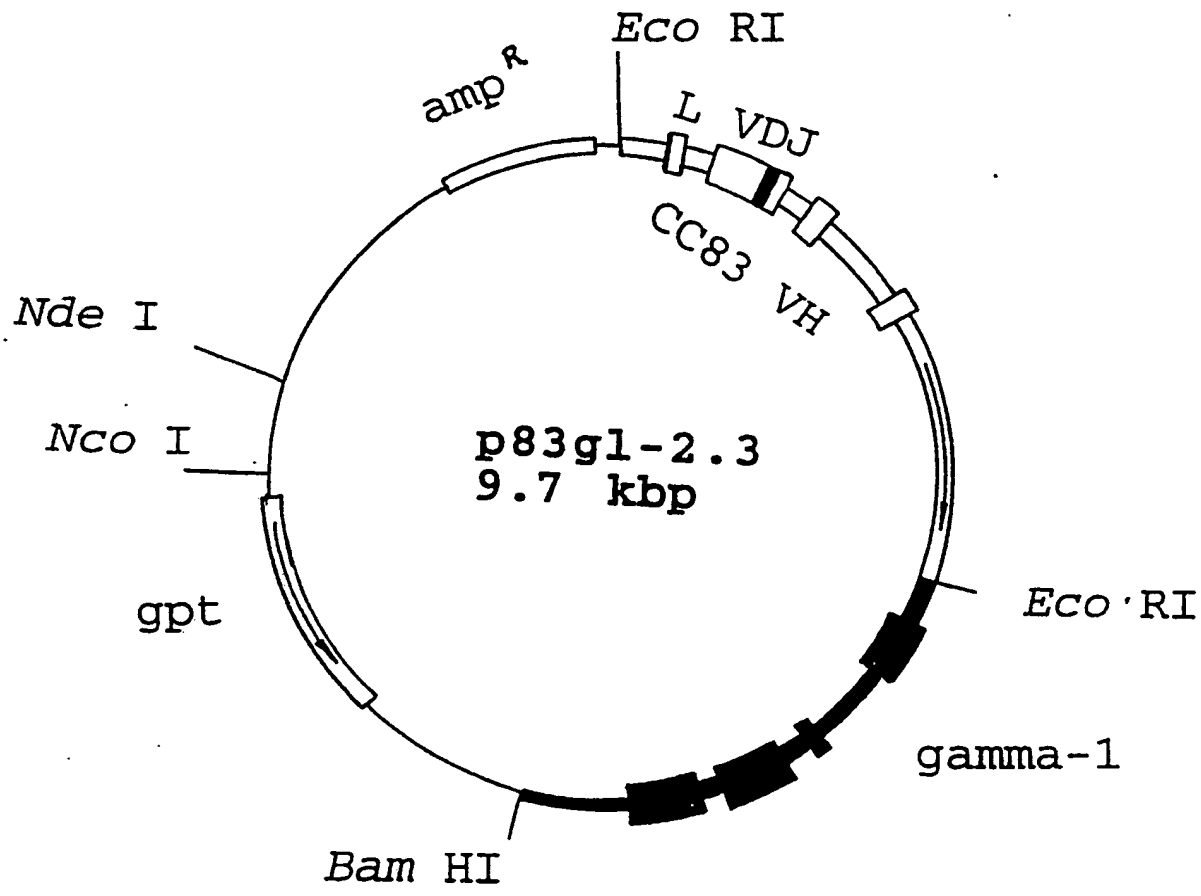

Eco RI
amp R
L
VDJ
CC83
VH
Nde I
Nco I
p83g1-2.3
9.7 kbp
gpt
Eco RI
gamma-1
Bam HI

FIG. 8

HUMVL (+), 26-MER:
(*Cla* I)
5'- GAAGAGT**ATC GAT**AAAATTT ATTGAG-3'

HUMVL(-), 98-MER:

(SPLICE SITE)→
(*Hind* III)
5'-CATT**AAGCTT** AGAAAAGTGT ACTTACGTTT
GATCACCACC TTGGTCCCTC CGCCGAAAGT
GAGAGGATAA CTATAATATT GCTGACAGTA
ATAAACTG-3'

FIG. 9

HJ4:

Leu Thr Phe Gly Gly Gly Thr Lys
CTC ACT TTC GGC GGA GGG ACC AAG

Val Glu Ile Lys A(rg)
GTG GAG ATC AAA C GTAAGTGCAC

TTTCCTAA

FIGURE 9: Human J4 (HJ4) amino acid and DNA sequences.The first two amino acids (Leu-Thr) complete the CDR3 region, the remainder make up the FR4 region. The (↓) indicates the splice site and the beginning of the intron between the J and C exons. DNA sequence underlined in HJ4 represents a part of the sequence used for the 3' end PCR oligo HUMVL(-).

FIG. 10


Cla I
5' ATCGATAAAA TTTATTGAGA ATTTGTTTAT TATGATTAAC 3418
3' TAGCTATTTT AAATAACTCT TAAACAAATA ATACTAATTG
AGAGGTAAAA GCCAGTATAT TACTGATTAA TATAGGTAAA 3458
TCTCCATTTT CGGTCATATA ATGACTAATT ATATCCATTT
*
AGGCAGTTAA GAAATTGGGA ATGCTTTCTC TTCTGCTTTC 3498
TCCGTCAATT CTTTAACCCT TACGAAAGAG AAGACGAAAG
TTCTACGATG CACAAGGCGT TTCACATTTA TGCCCCTATG 3538
AAGATGCTAC GTGTTCCGCA AAGTGTAAAT ACGGGGATAC
AAAATTACTA GGCTGTCCTA GTCATTAGAT CTTTCAGCAG 3578
TTTTAATGAT CCGACAGGAT CAGTAATCTA GAAAGTCGTC
TTTGTAGTTT TAGAGCTTCT AAGTTGACTT CTGTCTTTTC 3618
AAACATCAAA ATCTCGAAGA TTCAACTGAA GACAGAAAAG
TATTCATACA ATTACACATT CTGTGATGAT ATTTTTGGCT 3658
ATAAGTATGT TAATGTGTAA GACACTACTA TAAAAACCGA
HUMLIN1(-)

FIG. 10 (CONT.)

```
CTTGATTTAC ATTGGGTACT TTCACAACCC ACTGCTCATG 3698
GAACTAAATG TAACCCATGA AAGTGTTGGG TGACGAGTAC

AAATTTGCTT TTGTACTACT GGTTGTTTTT GCATAGGCCC 3738
TTTAAACGAA AACATGATGA CCAACAAAAA CGTATCCGGG

CTCCAGGCCA CGACCAGGTG TTTGGATTTT ATAAACGGGC 3778
GAGGTCCGGT GCTGGTCCAC AAACCTAAAA TATTTGCCCG

CGTTTGCATT GTGAACTGAG CTACAACAGG CAGGCAGGGG 3818
GCAAACGTAA CACTTGACTC GATGTTGTCC GTCCGTCCCC

          Met Val Leu Gln Thr Gln Val Leu Ile -10
CAGCAAG ATG GTG TTG CAG ACC CAG GTC TTC ATT 3852
GTCGTTC TAC CAC AAC GTC TGG GTC CAG AAG TAA

Ser Leu Leu Leu Trp Ile Ser G Intron
TCT CTG TTG CTC TGG ATC TCT G GTGA GGAATTAAAA -4
AGA GAC AAC GAG ACC TAG AGA C CACT CCTTAATTTT 3888

AGTGCCACAG TCTTTTCAGA GTAATATCTG TGTAGAAATA
TCACGGTGTC AGAAAAGTCT CATTATAGAC ACATCTTTAT
```

HUMLIN2(-)

FIG. 10 (CONT.)

```
AAAAAAATTA AGATATAGTT GGAAATAATG ACTATTTCCA
TTTTTTTAAT TCTATATCAA CCTTTATTAC TGATAAAGGT
     Bam HI
ATATGGATCC AATTATCTGC TGACTTATAA TACTACTAGA
TATACCTAGG TTAATAGACG ACTGAATATT ATGATGATCT

AAGCAAATTT AAATGACATA TTTCAATTAT ATCTGAGACA
TTCGTTTAAA TTTACTGTAT AAAGTTAATA TAGACTCTGT

GCGTGTATAA GTTTATGTAT AATCATTGTC CATTACTGAC
CGCACATATT CAAATACATA TTAGTAACAG GTAATGACTG

TACAG
ATGTC
```

```
                +1
ly Ala Tyr Gly Asp Ile Val Met Thr Gln Ser          7
GT GCC TAC GGG GAC ATC GTG ATG ACC CAG TCT       4125
CA CGG ATG CCC CTG TAG CAC TAC TGG GTC AGA
```

FIG. 10 (CONT.)

```
Pro Asp Ser Leu Ala Val Ser Leu Gly Glu Arg Ala Thr 20
CCA GAC TCC CTG GCT GTG TCT CTG GGC GAG AGG GCC ACC4164
GGT CTG AGG GAC CGA CAC AGA GAC CCG CTC TCC CGG TGG

                    <-------------CDR1-------------

Ile Asn Cys | Lys Ser Ser Gln Ser Val Leu Tyr Ser Ser 27F
ATC AAC TGC | AAG TCC AGC CAG AGT GTT TTA TAC AGC TCC4203
TAG TTG ACG | TTC AGG TCG GTC TCA CAA AAT ATG TCG AGG

-------------------------->

Asn Asn Lys Asn Tyr Leu Ala | Trp Tyr Gln Gln Lys Pro 40
AAC AAT AAG AAC TAC TTA GCT | TGG TAC CAG CAG AAA CCA4242
TTG TTA TTC TTG ATG AAT CGA | ACC ATG GTC GTC TTT GGT

HUMLCDR1(-)                                <-----CDR2-----

Gly Gln Pro Pro Lys Leu Leu Ile Tyr | Trp Ala Ser Thr 53
GGA CAG CCT CCT AAG CTG CTC ATT TAC | TGG GCA TCT ACC4281
CCT GTC GGA GGA TTC GAC GAG TAA ATG | ACC CGT AGA TGG

------------->

Arg Glu Ser | Gly Val Pro Asp Arg Phe Ser Gly Ser Gly 66
CGG GAA TCC | GGG GTC CCT GAC CGA TTC AGT CGC AGT CGC4320
GCC CTT AGG | CCC CAG GGA CTG GCT AAG TCA CCG TCG CCC

Ser Gly Thr Asp Phe Thr | Leu | Thr Ile Ser Ser Leu Gln Ala
TCT GGG ACA GAT TTC ACT | CTC | ACC ATC AGC AGC CTG CAG GCT
AGA CCC TGT CTA AAG TGA | GAG | TGG TAG TCG TCG GAC GTC CGA
```

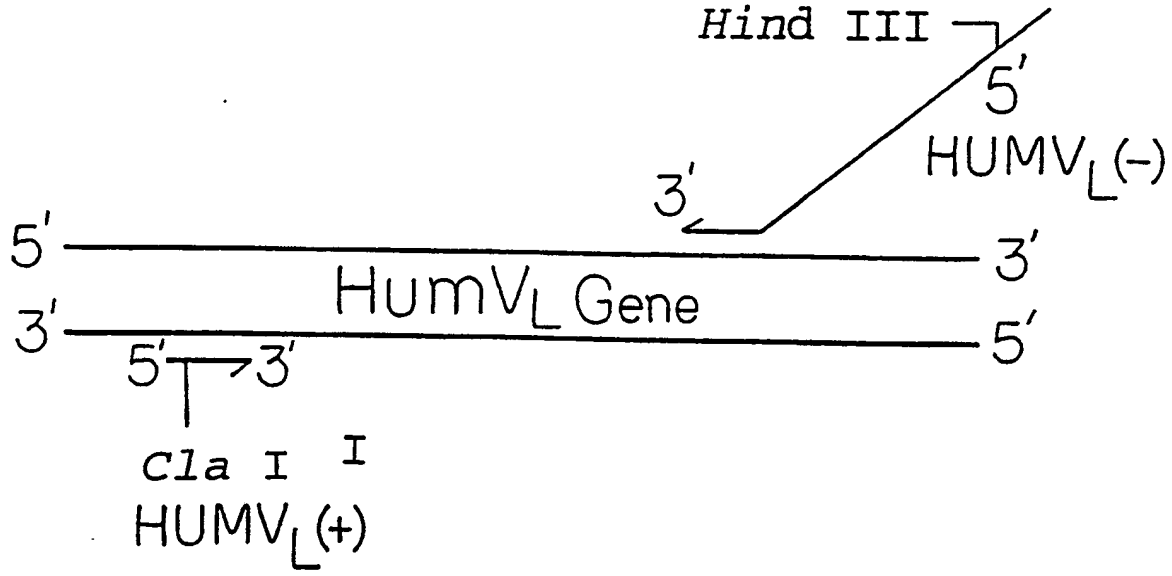
FIG. 10 (CONT.)

```
                                                    |<-------- CDR3 ------
Glu Asp Val Ala Val Tyr Tyr Cys | Gln Gln Tyr Tyr Ser 93
GAA GAT GTG GCA GTT TAT TAC TGT | CAG CAA TAT TAT AGT4400
CTT CTA CAC CGT CAA ATA ATG ACA | GTC GTT ATA ATA TCA
                               |
------------------------------>|
Tyr Pro Leu Thr | Phe Gly Gly Gly Thr Lys Val Val Ile 106
TAT CCT CTC ACT | TTC GGC GGA GGG ACC AAG GTG GTG AAA4440
ATA GGA GAG TGA | AAG CCG CCT CCC TGG TTC CAC CAC TAG
               |
Lys A(rg)                    Hind III                107
AAA C GTAAGTACAC TTTTCTAAG CTT-3'                    4466
TTT G CATTCATGTG AAAAGATTC GAA 5'
```

FIG. 10 (CONT.)

FIGURE 10: Entire DNA sequence of the Hum4 V gene *Cla* I-*Hin*d III segment in pRL1001, Clone #2. A single base difference occured at position 3461 and is marked with an asterisk (*). The corresponding amino acid sequences in the coding exons are shown. The site of the Leu-Pro mutation in Clone #7 is boxed. An arrow () indicates the site of the single base deletion in Clone #11. Oligonucleotides used as primers for the sequencing reactions are underlined. In order as they occur from the 5' end are: HUMLIN1(-), HUMLIN2(-), HUMLCDR1(-) and *Hin*d III Ck(-)(not shown).

FIG. 11



A schematic representation of the human germline Subgroup IV gene (HumV_L) as the target for the PCR. The 5'-end oligo (HUMVL(+)) and the 3'end oligo (HUMVL(-)) used to prime the elongation reactions for Taq polymerase are shown with half-arrows to indicate the direction of synthesis.

FIG. 12

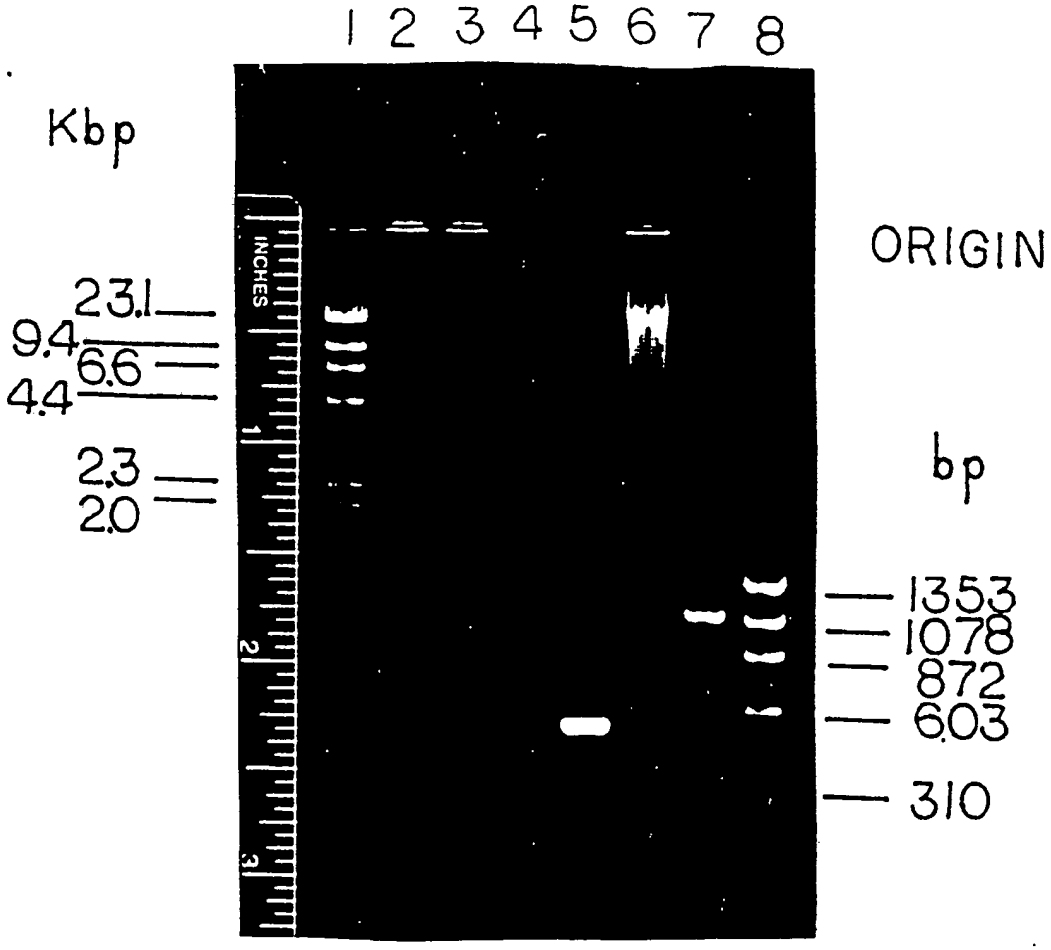


FIGURE 12: Agarose gel electrophoresis of Hum4V$_L$ PCR reactions. Lane 1: λ *Hind* III standard; lane 2: no Taq polymerase control; lane 3: no primers added; lane 4: no human DNA template; lane 5: Gene *Amp* kit positive control; lane 6: 3 μg human DNA with primers and Taq polymerase; lane 7: same as lane 6, but with 1ug human DNA and lane 8: ØX174-*Hae* III DNA standard. Ethidium bromide was added to the gel and buffer. Bands were visualized by long wavelength UV light.

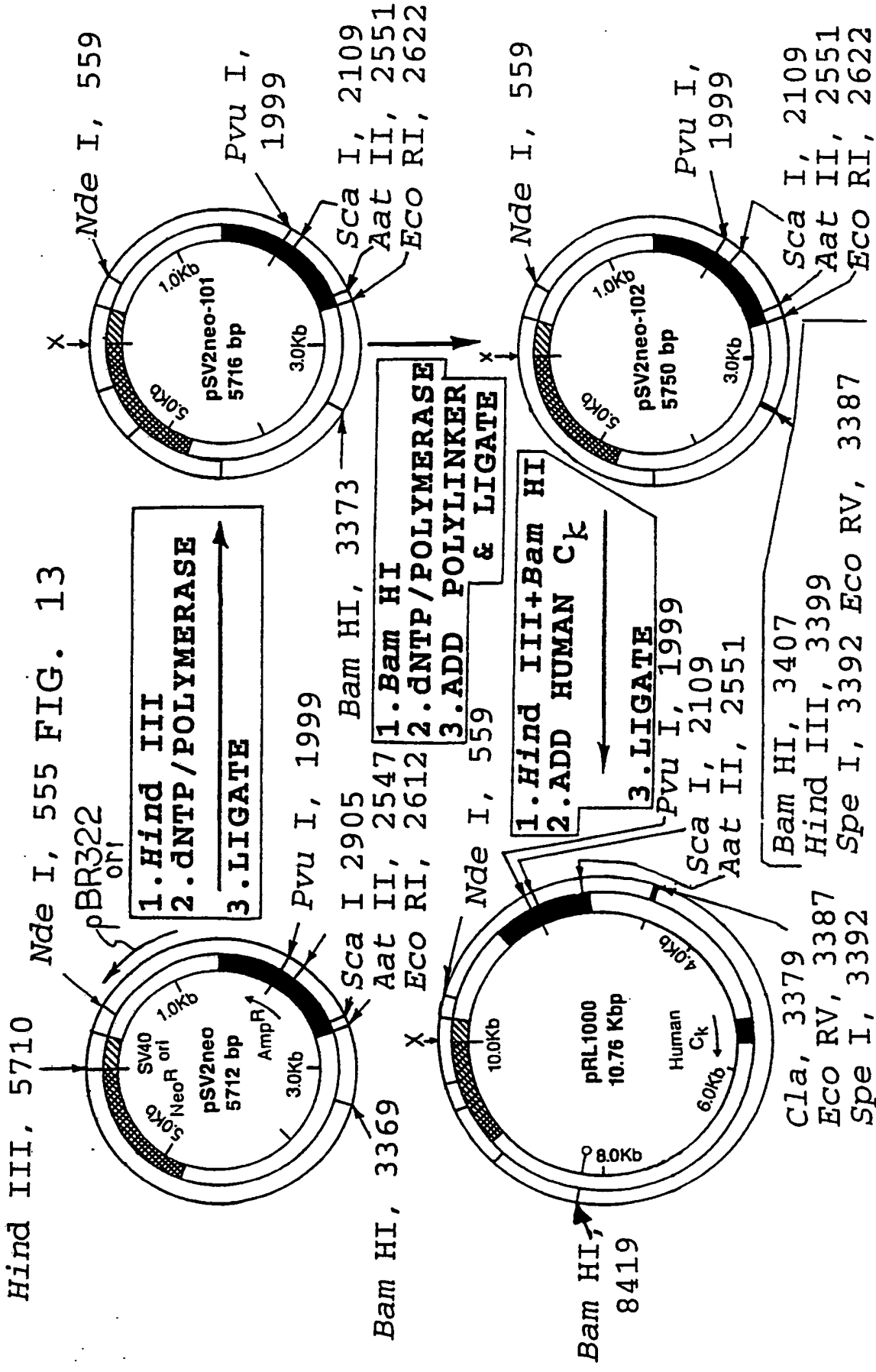

Hind III, 5710
Nde I, 555
pBR322 ori
SV40 ori
NeoR
pSV2neo 5712 bp
1.0Kb
3.0Kb
5.0Kb
AmpR
1.Hind III
2.dNTP/POLYMERASE
3.LIGATE
Pvu I, 1999
Sca I 2905
Aat II, 2547
Eco RI, 2612
Bam HI, 3369
pSV2neo-101 5716 bp
Nde I, 559
Pvu I, 1999
Sca I, 2109
Aat II, 2551
Eco RI, 2622
Bam HI, 3373
1.Bam HI
2.dNTP/POLYMERASE
3.ADD POLYLINKER & LIGATE
pRL1000 10.76 Kbp
10.0Kb
8.0Kb
4.0Kb
6.0Kb
Human Ck
Bam HI, 8419
1.Hind III+Bam HI
2.ADD HUMAN Ck
3.LIGATE
Pvu I, 1999
Sca I, 2109
Aat II, 2551
Nde I, 559
pSV2neo-102 5750 bp
Bam HI, 3407
Hind III, 3399
Spe I, 3392
Cla I, 3379
Eco RV, 3387

FIG. 13

FIG. 14

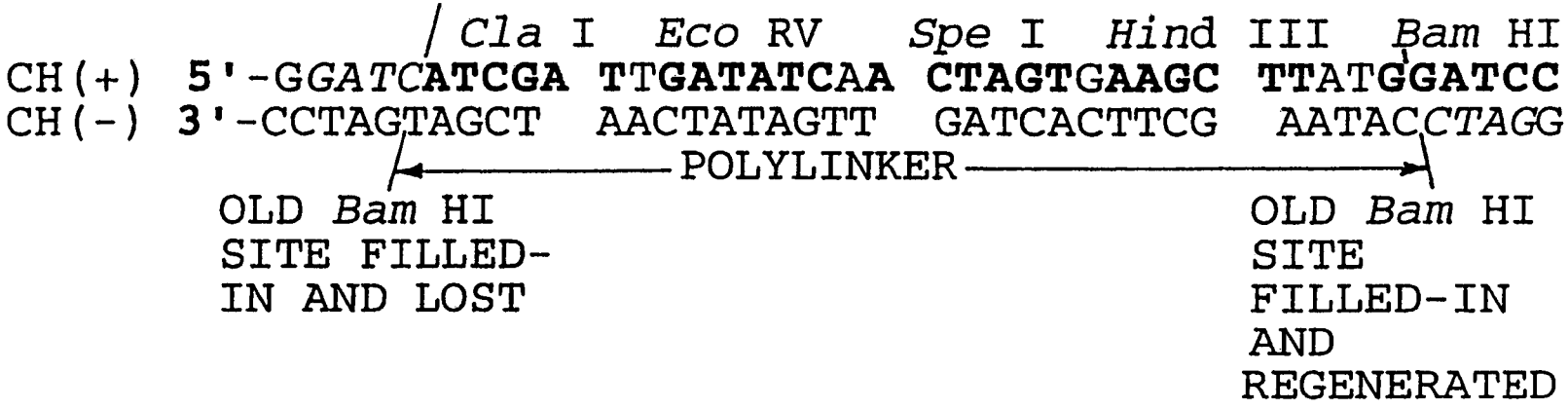


FIGURE 14: The polylinker inserted between filled-in *Bam* HI site of pSV2neo-101 to create pSV2neo-102. Note that the polylinker could be inserted in both orientations such that the *Bam* HI site on the left side could also be regenerated (and the one on the right side lost). The nucleotides used to fill-in the *Bam* HI site are shown in italics. The top synthetic oligo was called CH(+) while the complimentary strand was CH(-).

FIG. 15

(A portion of the DNA
Sequence of pSV2neo)

←——TOWARDS *Eco* RI SITE 5'-GAGGAGGTTA

GGGTTTATGA GGACACAGAG GAGCTTCCTG

G**GGATCC**AGA CATGATAAGA TACATTGATG
Bam H1

AGTTTGGACA AACCACAACT AGA-3'

FIGURE 15: Oligonucleotide synthesized (21-mer, called NEO102SEQ) to sequence putative pSV2neo-102 clones is the underlined sequence shown above. The *Bam* HI site where the polylinker was inserted in pSV2neo-101 is boxed.

FIG. 16

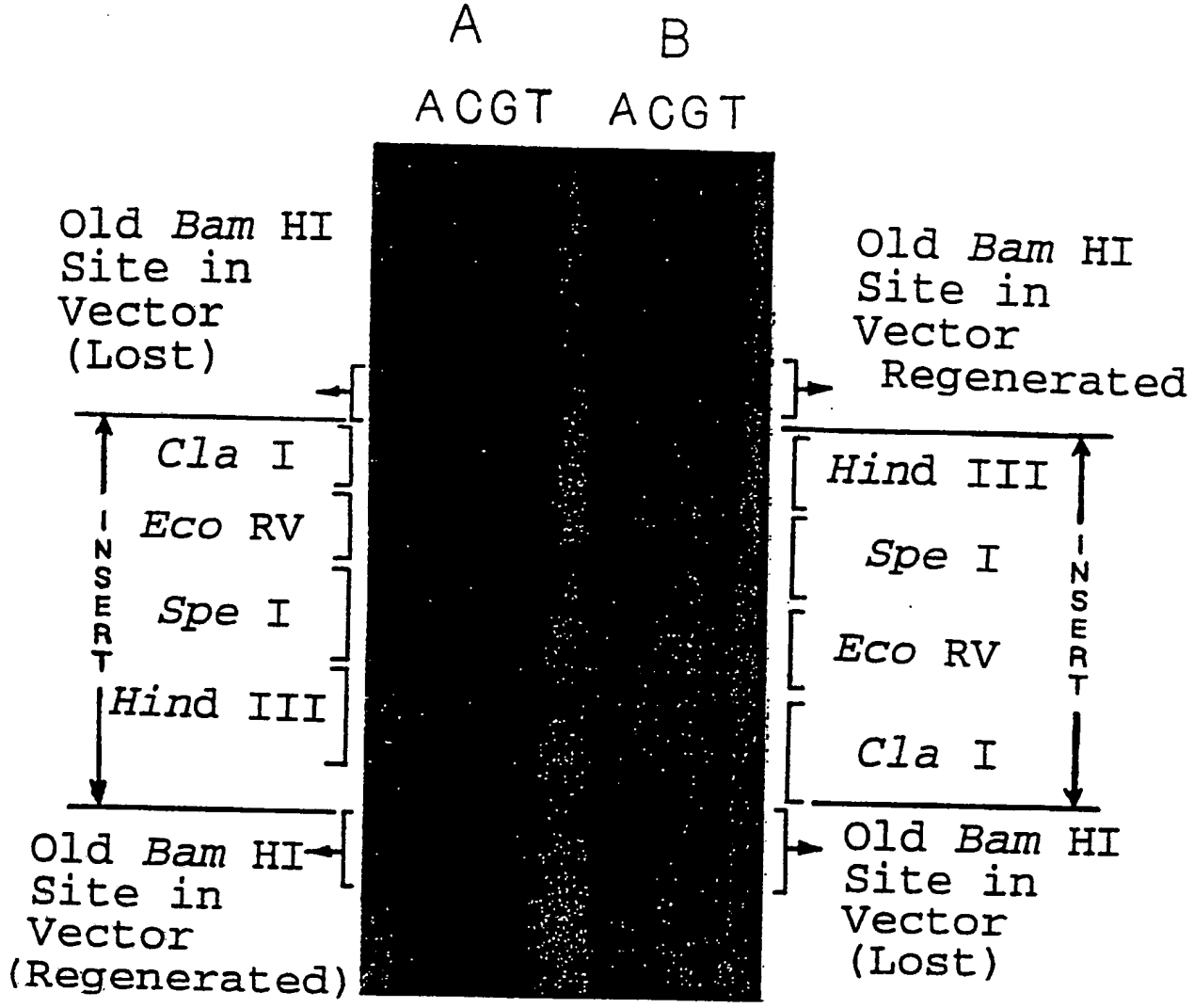


FIGURE 16: P-32 autoradiogram showing polylinker DNA sequence cloned in *Bam* HI site of pSV2neo-101. In both cases a single 30-base linker unit was incorporated, but in opposite orientations. Panel A-Sequence resulting in pSV2neo-120; Panel B-sequence resulting in pSV2neo-102. Reading the sequence (going up) is in the 5' to 3' direction of the (+) strand.

FIG. 17

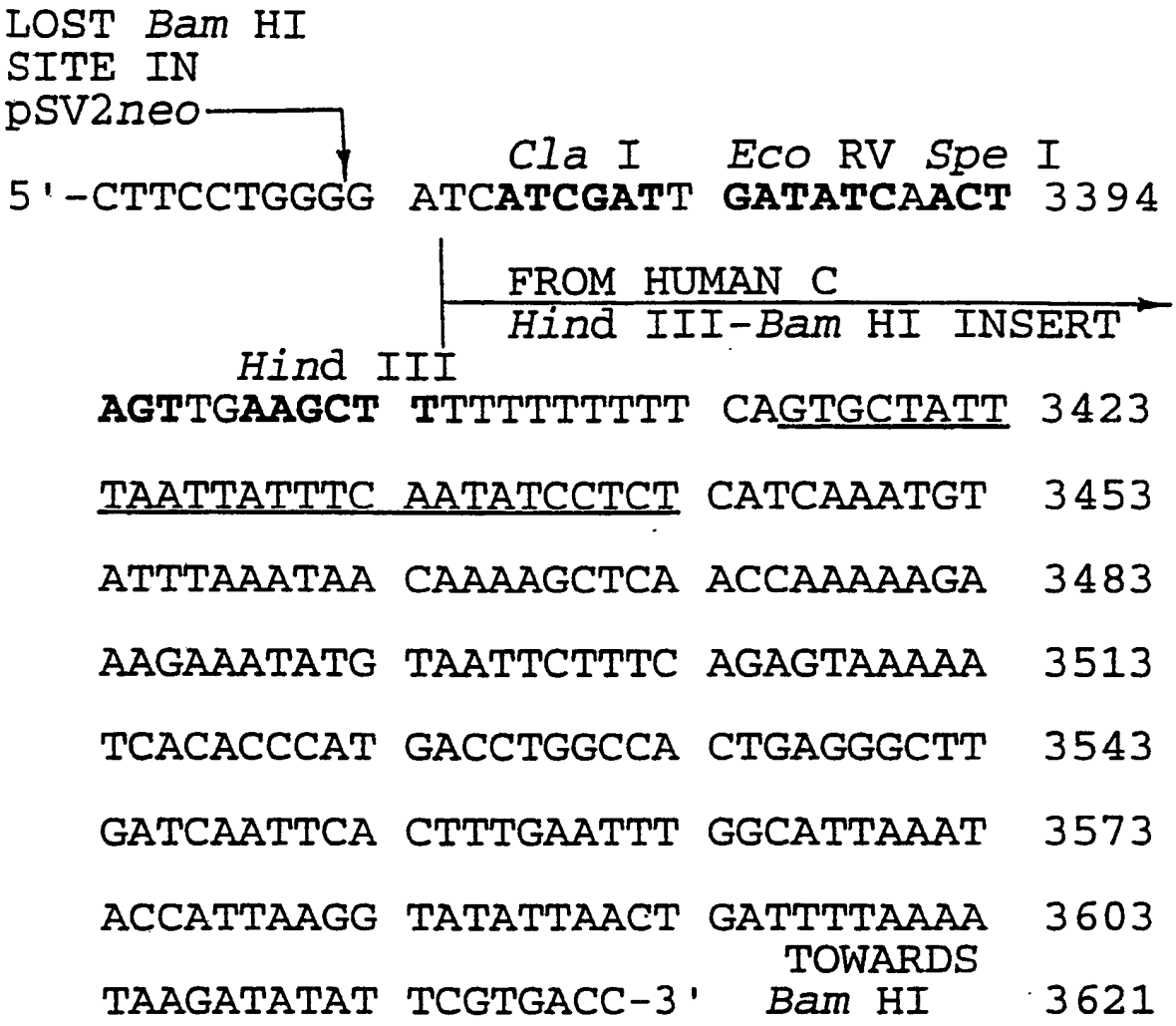


FIGURE 17: DNA sequence from pRL1000, reading the (+) strand from the primer NEO102SEQ (Figure 15). Sequence data past the *Hind* III site is from the human C_K Hind III - *Bam* HI insert. The sequence complementary to the underlined DNA sequence, called *Hind* IIIC_K(-), was synthesized as a primer for sequencing in the upstream 3' direction.

FIG. 18

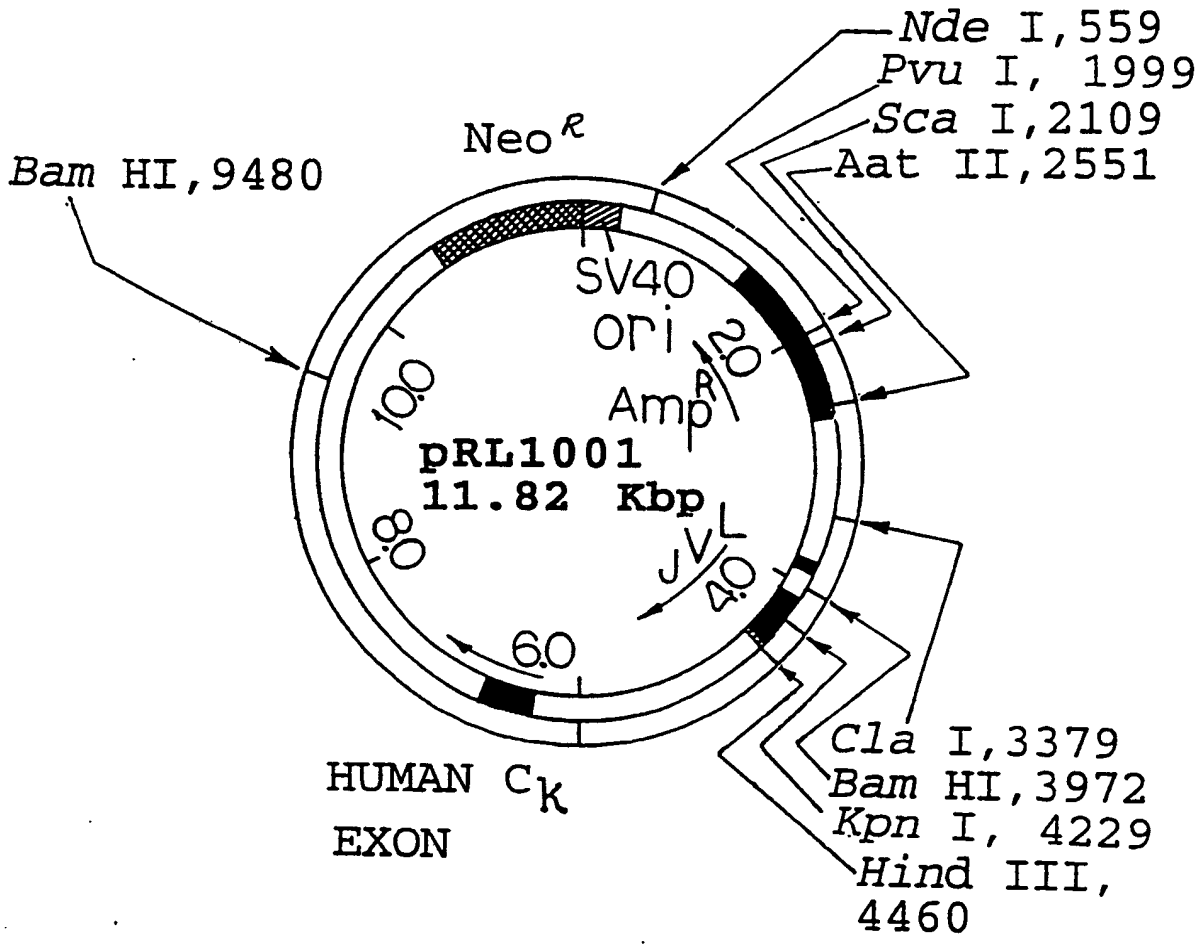


FIGURE 18: Partial restriction map of the plasmid pRL1001. This is the expression vector to introduce the human anti-tumor L chain gene in Sp2/0 cells.

FIG. 19

DNA SEQUENCING – pRL1001

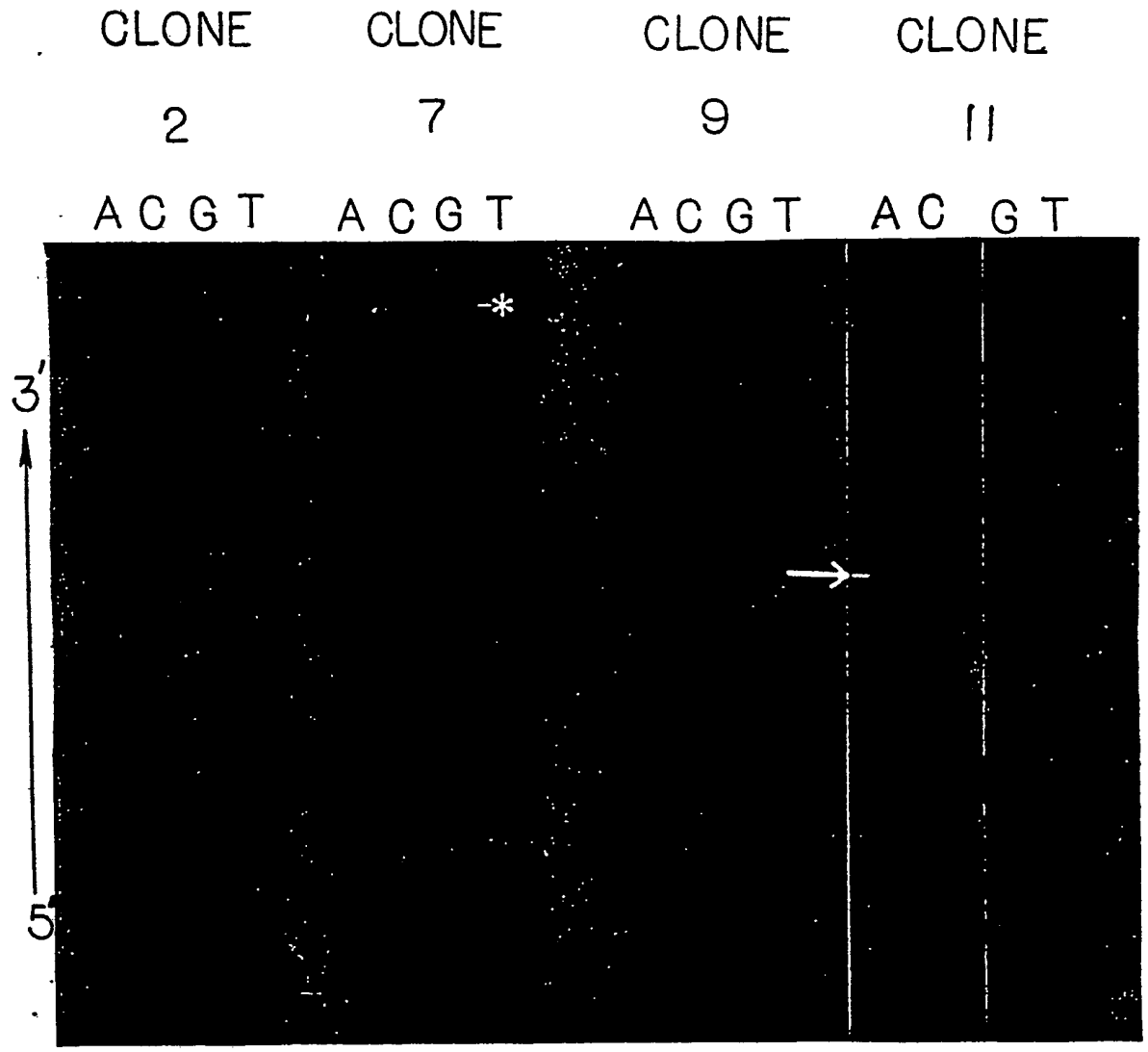
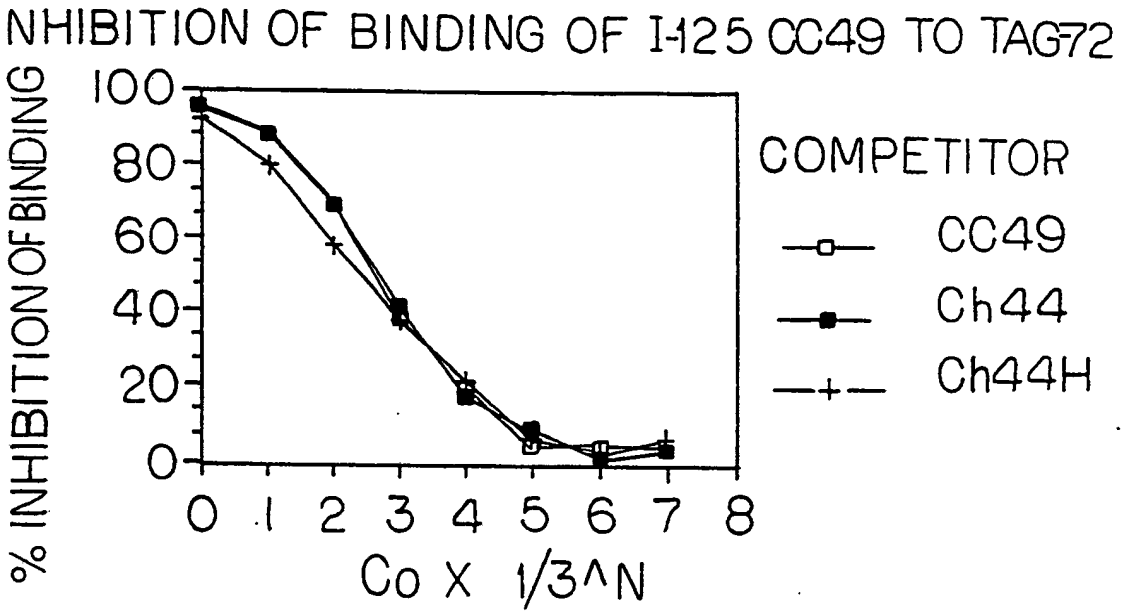


FIGURE 19: DNA sequence autoradiogram of pRL1001 clones. Reading the gel is in the 5' to 3' direction on the (-) strand, from the *Hind* III C_K(-) primer. Clones 2 and 9 were equivalent to the expected sequence, clone 7 had a single base substitution (marked by *) and clone 11 had a single base deletion (marked by -)

Fig. 20

RECIPROCAL COMPETITION BETWEEN CC49, Ch44, AND Ch44H



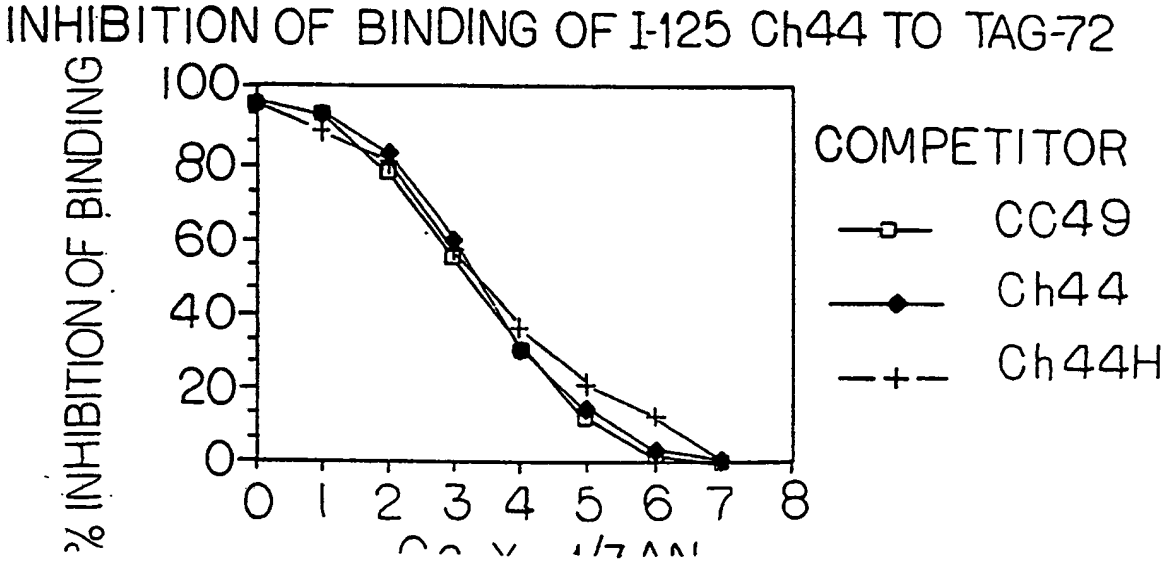


Fig. 20 (CONT.)

RECIPROCAL COMPETITION BETWEEN

CC49, Ch44, AND Ch44H

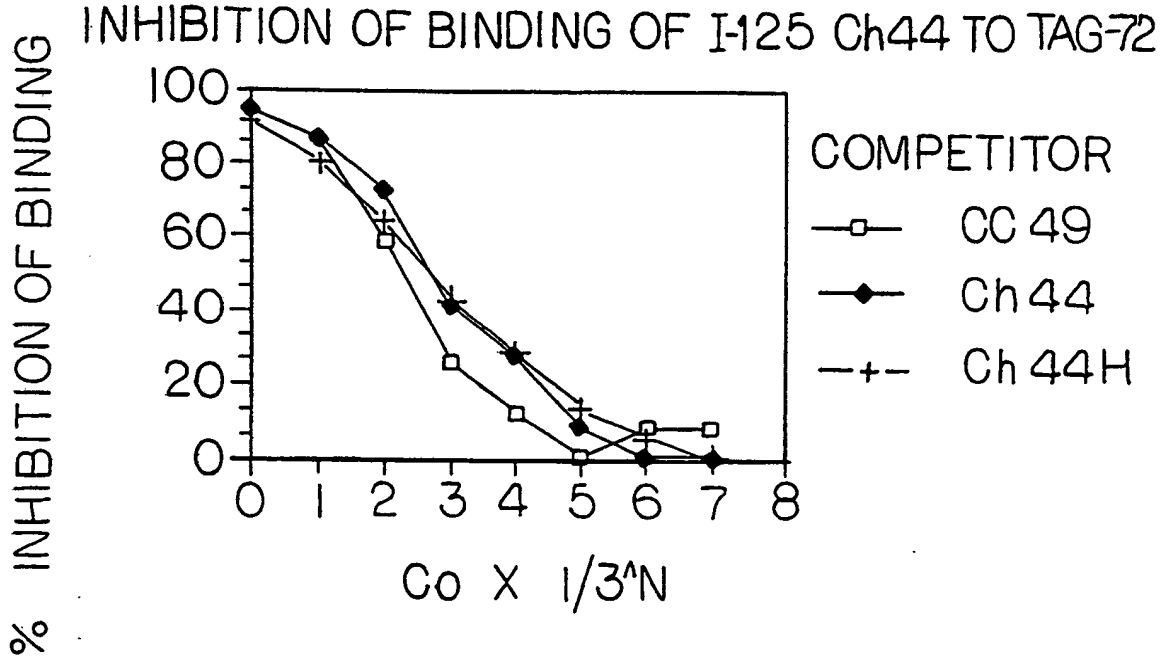


FIG. 21

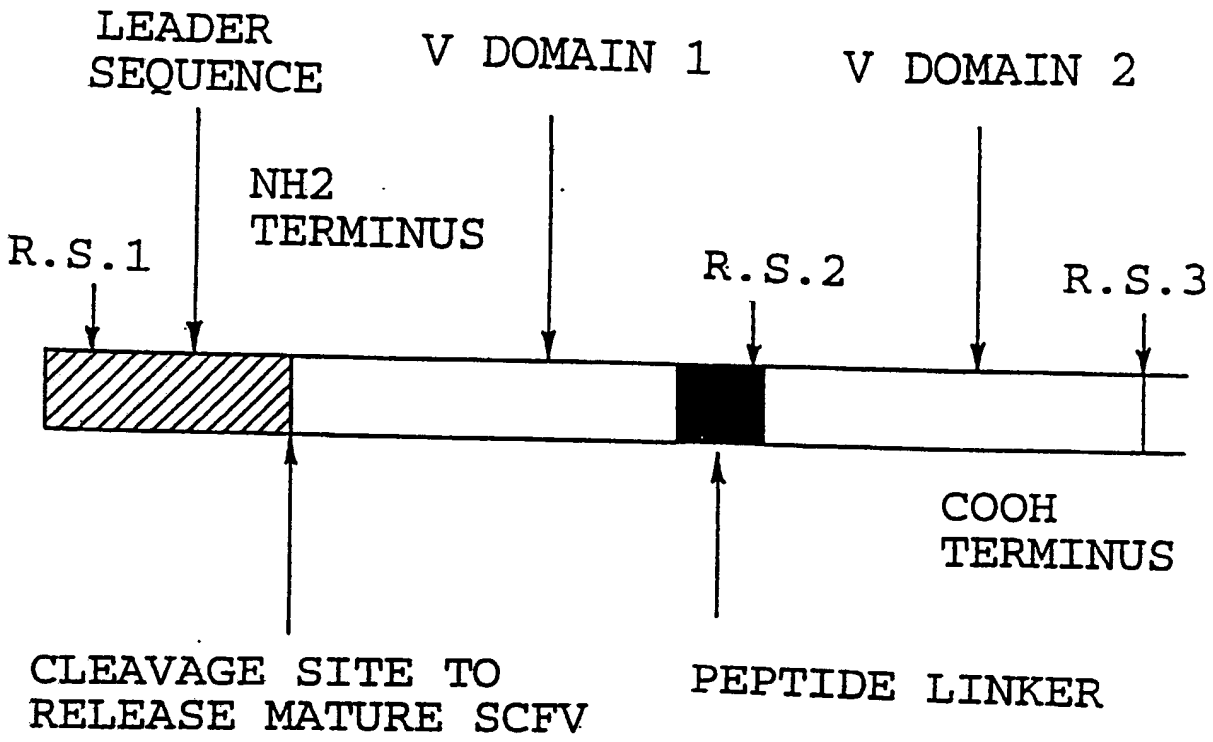

LEADER SEQUENCE
V DOMAIN 1
V DOMAIN 2
NH2 TERMINUS
R.S.1
R.S.2
R.S.3
COOH TERMINUS
CLEAVAGE SITE TO RELEASE MATURE SCFV
PEPTIDE LINKER

FIG. 22

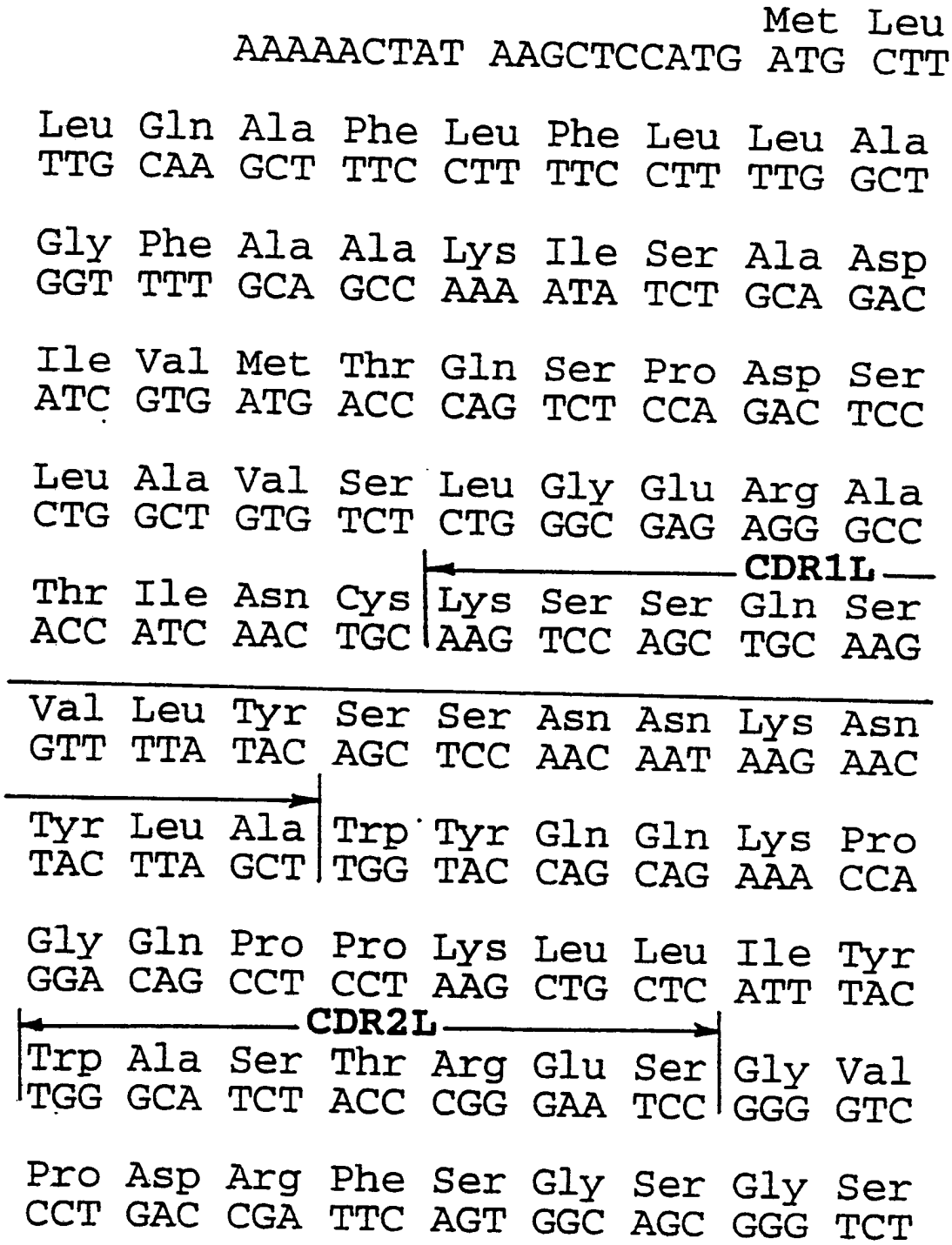

Met Leu
AAAAACTAT AAGCTCCATG ATG CTT
Leu Gln Ala Phe Leu Phe Leu Leu Ala
TTG CAA GCT TTC CTT TTC CTT TTG GCT
Gly Phe Ala Ala Lys Ile Ser Ala Asp
GGT TTT GCA GCC AAA ATA TCT GCA GAC
Ile Val Met Thr Gln Ser Pro Asp Ser
ATC GTG ATG ACC CAG TCT CCA GAC TCC
Leu Ala Val Ser Leu Gly Glu Arg Ala
CTG GCT GTG TCT CTG GGC GAG AGG GCC
CDR1L
Thr Ile Asn Cys Lys Ser Ser Gln Ser
ACC ATC AAC TGC AAG TCC AGC TGC AAG
Val Leu Tyr Ser Ser Asn Asn Lys Asn
GTT TTA TAC AGC TCC AAC AAT AAG AAC
Tyr Leu Ala Trp Tyr Gln Gln Lys Pro
TAC TTA GCT TGG TAC CAG CAG AAA CCA
Gly Gln Pro Pro Lys Leu Leu Ile Tyr
GGA CAG CCT CCT AAG CTG CTC ATT TAC
CDR2L
Trp Ala Ser Thr Arg Glu Ser Gly Val
TGG GCA TCT ACC CGG GAA TCC GGG GTC
Pro Asp Arg Phe Ser Gly Ser Gly Ser
CCT GAC CGA TTC AGT GGC AGC GGG TCT

FIG. 22 (CONT.)

Gly Thr Asp Phe Thr Leu Thr Ile Ser
GGG ACA GAT TTC ACT CTC ACC ATC AGC

Ser Leu Gln Ala Glu Asp Val Ala Val
AGC CTG CAG GCT GAA GAT GTG GCA GTT

CDR3L

Tyr Tyr Cys | Gln Gln Tyr Tyr Ser Tyr
TAT TAC TGT | CAG CAA TAT TAT AGT TAT

Pro Leu Thr | Phe Gly Gly Gly Thr Lys
CCT CTC ACT | TTC GGC GGA GGG ACC AAG

Val | Lys Glu Ser Gly Ser Val Ser Ser
GTG | AAG GAG TCA GGT TCG GTC TCC TCA

LINKER

Glu Gln Leu Ala Gln Phe Arg Ser Leu
GAA CAA TTG GCC CAA TTT CGT TCC TTA

Asp | Val Gln Leu Gln Gln Ser Asp Ala
GAC | GTC CAG TTG CAG CAG TCT GAC GCT

Glu Leu Val Lys Pro Gly Ala Ser Val
GAG TTG GTG AAA CCT GGG GCT TCA GTG

Lys Ile Ser Cys Lys Ala Ser Gly Tyr
AAG ATT TCC TGC AAG GCT TCT GGC TAC

CDR1H

Thr Phe Thr | Asp His Ala Ile His | Trp
ACC TTC ACT | GAC CAT GCA ATT CAC | TGG

Val Lys Gln Asn Pro Glu Gln Gly Leu
GTG AAA CAG AAC CCT GAA CAG GGC CTG

FIG. 22 (CONT.)

Glu	Trp	Ile	Gly	Tyr	Phe	Ser	Pro	Gly
GAA	TGG	ATT	GGA	TAT	TTT	TCT	CCC	GGA
				CDR2H				
Asn	Asp	Asp	Phe	Lys	Tyr	Asn	Glu	Arg
AAT	GAT	GAT	TTT	AAA	TAC	AAT	GAG	AGG
Phe	Lys	Gly	Lys	Ala	Thr	Leu	Thr	Ala
TTC	AAG	GGC	AAG	GCC	ACA	CTG	ACT	GCA
Asp	Lys	Ser	Ser	Ser	Thr	Ala	Tyr	Val
GAC	AAA	TCC	TCC	AGC	ACT	GCC	TAC	GTG
Gln	Leu	Asn	Ser	Leu	Thr	Ser	Glu	Asp
CAG	CTC	AAC	AGC	CTG	ACA	TCT	GAG	GAT
Ser	Ala	Val	Tyr	Phe	Cys	Thr	Arg	Ser
TCT	GCA	GTG	TAT	TTC	TGT	ACA	AGA	TCC
	CDR3H							
Leu	Asn	Met	Ala	Tyr	Trp	Gly	Gln	Gly
CTG	AAT	ATG	GCC	TAC	TGG	GGT	CAA	GGA
Thr	Ser	Val	Thr	Val	Ser			
ACC	TCA	GTC	ACC	GTC	TCC	TAG	TGA	

AGCTTGGAAC ACCACACAAA CCATATCCAA A

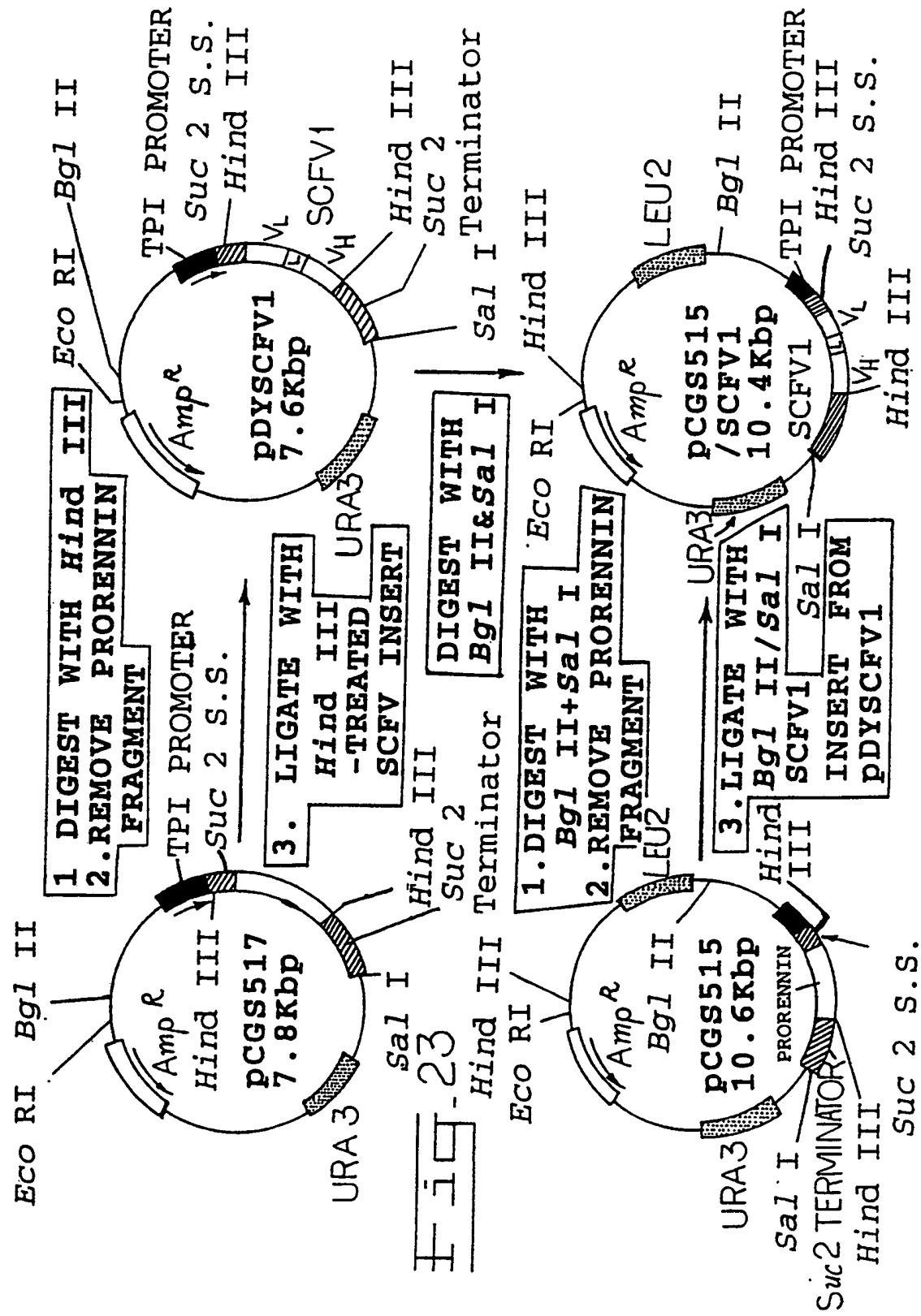

pCGS517
7.8Kbp
Amp R
Eco RI
Bgl II
Hind III
URA3
Sal I
Hind III
Suc 2 Terminator
TPI PROMOTER
Suc 2 S.S.
1 DIGEST WITH Hind III
2.REMOVE PRORENNIN FRAGMENT
3. LIGATE WITH Hind III -TREATED SCFV INSERT
pDYSCFV1
7.6Kbp
Amp R
Eco RI
Bgl II
TPI PROMOTER
Suc 2 S.S.
Hind III
VL
SCFV1
VH
Hind III
Suc 2 Terminator
Sal I
DIGEST WITH Bgl II&Sal I
1.DIGEST WITH Bgl II+Sal I
2.REMOVE PRORENNIN FRAGMENT
pCGS515
10.6Kbp
Amp R
Bgl II
LEU2
URA3
Sal I
Suc2 TERMINATOR
PRORENNIN
Hind III
Suc 2 S.S.
3.LIGATE WITH Bgl II/Sal I
SCFV1
Sal I
INSERT FROM pDYSCFV1
pCGS515
/SCFV1
10.4Kbp
Amp R
Bgl II
LEU2
TPI PROMOTER
Hind III
VL
SCFV1
VH
Hind III
Suc 2 S.S.

Fig-23

Fig. 24

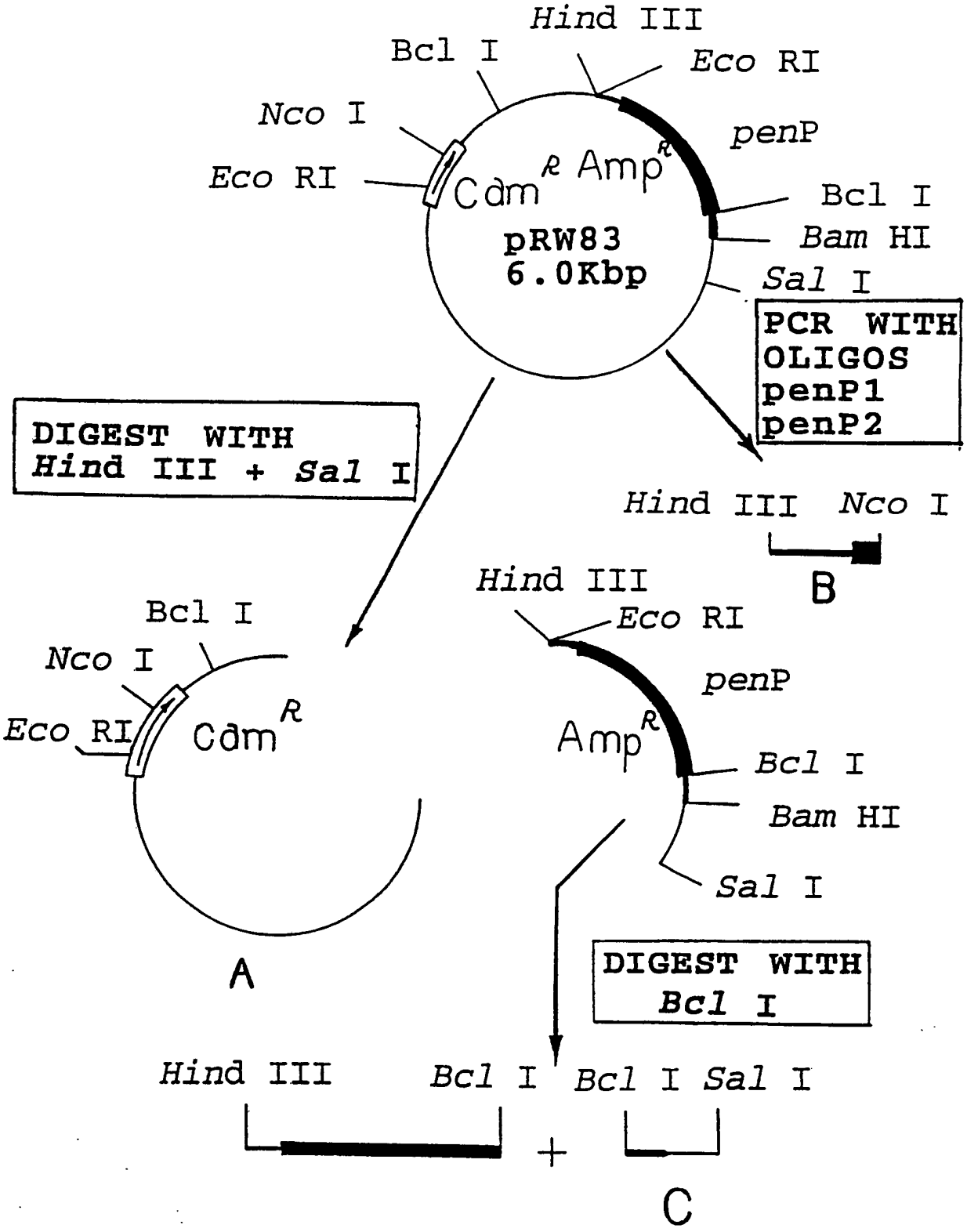
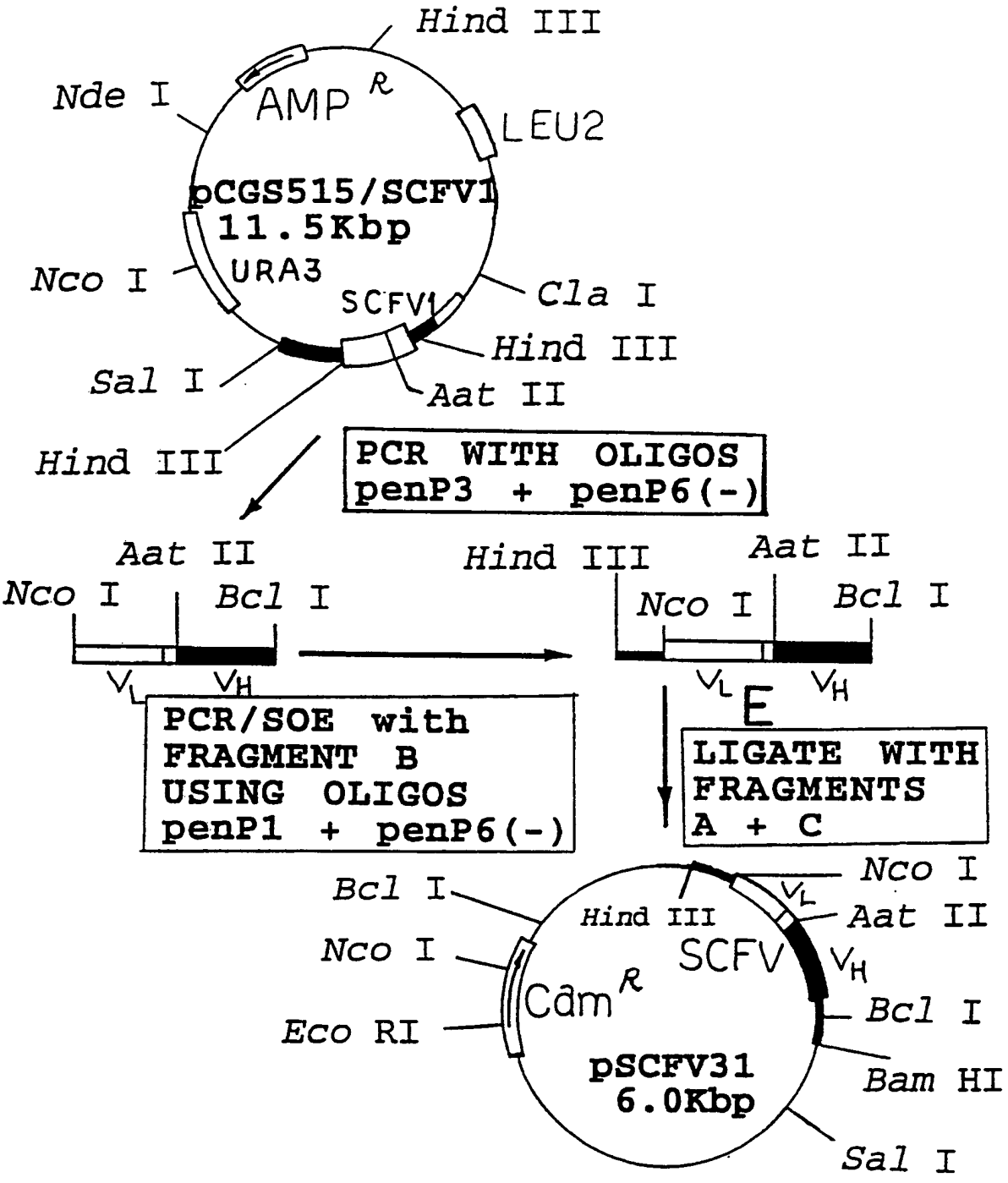

Hind III
Bcl I
Eco RI
Nco I
penP
Eco RI
Cam R Amp R
Bcl I
pRW83
6.0Kbp
Bam HI
Sal I
PCR WITH
OLIGOS
penP1
penP2
DIGEST WITH
Hind III + Sal I
Hind III Nco I
B
Hind III
Eco RI
Bcl I
Nco I
penP
Eco RI
Cam R
Amp R
Bcl I
Bam HI
Sal I
A
DIGEST WITH
Bcl I
Hind III
Bcl I
Bcl I Sal I
+
C

Fig. 24 (CONT.)


Hind III
Nde I
AMP R
LEU2
pCGS515/SCFV1
11.5Kbp
Nco I
URA3
SCFV
Cla I
Hind III
Sal I
Aat II
Hind III
PCR WITH OLIGOS
penP3 + penP6(-)
Aat II
Nco I
Bcl I
VL
VH
Hind III
Aat II
Nco I
Bcl I
VL
VH
E
PCR/SOE with
FRAGMENT B
USING OLIGOS
penP1 + penP6(-)
LIGATE WITH
FRAGMENTS
A + C
Bcl I
Nco I
Eco RI
Hind III
VL
Nco I
Aat II
SCFV
VH
Cam R
Bcl I
pSCFV31
6.0Kbp
Bam HI
Sal I

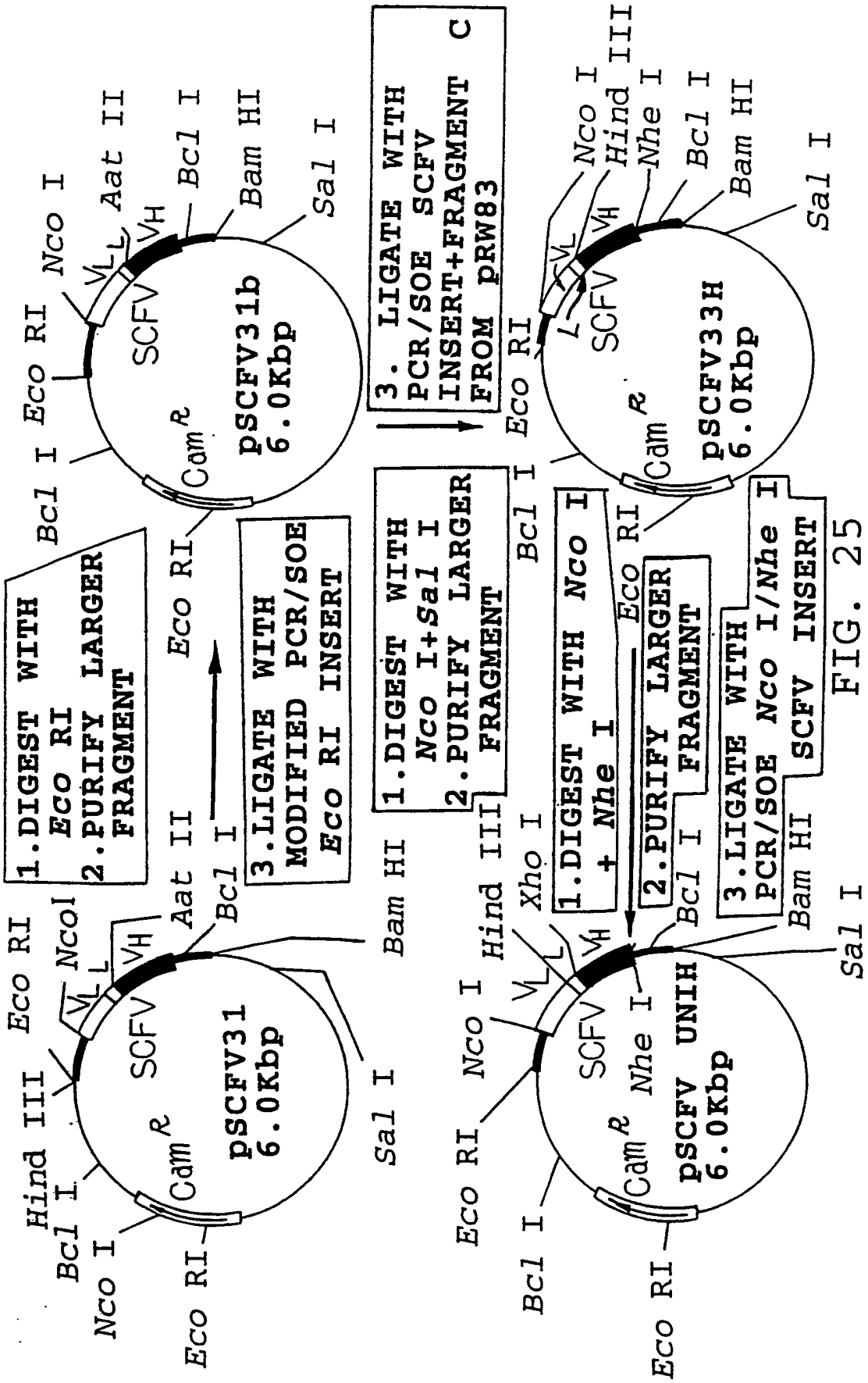

pSCFV31
6.0Kbp
pSCFV31b
6.0Kbp
pSCFV33H
6.0Kbp
pSCFV UNIH
6.0Kbp
SCFV
Cam R
1.DIGEST WITH Eco RI
2.PURIFY LARGER FRAGMENT
3.LIGATE WITH MODIFIED PCR/SOE Eco RI INSERT
1.DIGEST WITH Nco I+Sal I
2.PURIFY LARGER FRAGMENT
3. LIGATE WITH PCR/SOE SCFV INSERT+FRAGMENT C FROM pRW83
1.DIGEST WITH Nco I + Nhe I
2.PURIFY LARGER FRAGMENT
3.LIGATE WITH PCR/SOE Nco I/Nhe I SCFV INSERT
Eco RI
Nco I
Hind III
Bcl I
Aat II
Bam HI
Sal I
Xho I
Nhe I

FIG. 25

FIG. 26

Eco **RI**

CTCATGTTTG ACAGCTTATC ATCGAT**GAAT**

TCCATCACTT CCCTCCGTTC ATTTGTCCCC

GGTGGAAACG AGGTCATCAT TTCCTTCCGA

AAAAACGGTT GCATTTAAAT CTTACATATG

TAATACTTTC AAAGACTACA TTTGTAAGAT

TTGATGTTTG AGTCGGCTGA AAGATCGTAC

GTACCAATTA TTGTTTCGTG ATTGTTCAAG

CCATAACACT GTAGGGATAG TGGAAAGAGT

GCTTCATCTG GTTACGATCA ATCAAATATT

pelB **Signal**

	Met	Lys	Tyr	Leu
CAAACGGAGG GAGACGATTT TG|ATG	AAA	TAC	CTA

Sequence

Leu	Pro	Thr	Ala	Ala	Ala	Gly	Leu	Leu	Leu
TTG	CCT	ACG	GCA	GCC	GCT	GGA	TTG	TTA	TTA

Nco **I** → ← **H4V$_L$**

Leu	Ala	Ala	Gln	Pro	Ala	Met	Ala|	Asp	Ile
CTC	GCT	GCC	CAA	CCA	**GCC**	**ATG**	GCC|	GAC	ATC

Val	Met	Thr	Gln	Ser	Pro	Asp	Ser	Leu	Ala
GTG	ATG	ACC	CAG	TCT	CCA	GAC	TCC	CTG	GCT

Val	Ser	Leu	Gly	Glu	Arg	Ala	Thr	Ile	Asn
GTG	TCT	CTG	GGC	GAG	AGG	GCC	ACC	ATC	AAC

Cys	Lys	Ser	Ser	Gln	Ser	Val	Leu	Tyr	Ser
TGC	AAG	TCC	AGC	CAG	AGT	GTT	TTA	TAC	AGC

FIG. 26 (CONT.)

Ser Asn Asn Lys Asn Tyr Leu Ala Trp Tyr
TCC AAC AAT AAG AAC TAC TTA GCT TGG TAC

Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu
CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG

Leu Ile Tyr Trp Ala Ser Thr Arg Glu Ser
CTC ATT TAC TGG GCA TCT ACC CGG GAA TCC

Gly Val Pro Asp Arg Phe Ser Gly Ser Gly
GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG

Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC

Ser Leu Gln Ala Glu Asp Val Ala Val Tyr
AGC CTG CAG GCT GAA GAT GTG GCA GTT TAT

Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu
TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC

$H4V_L$

Thr Phe Gly Gly Gly Thr Lys Val Val Ile
ACT TTC GGC GGA GGG ACC AAG GTG GTG ATC

***Hind* III LINKER**

Lys | Leu Ser Ala Asp Asp Ala Lys Lys Asp
AAG | **CTT** AGT GCG GAC GAT GCG AAA AAG GAT

Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp
GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC

LINKER *Xho* I CC49 V_H

Asp Ala Lys Lys Asp Leu | Glu Val Gln Leu
GAT GCT AAA AAG GAC **CTC** | **GAG** GTT CAG TTG

FIG. 26 (CONT.)

```
Gln Gln Ser Ala Glu Leu Val Lys Pro Gly
CAG CAG TCT GCT GAG TTG GTG AAA CCT GGG

Ala Ser Val Lys Ile Ser Cys Lys Ala Ser
GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT

Gly Tyr Thr Phe Thr Asp His Ala Ile His
GGC TAC ACC TTC ACT GAC CAT GCA ATT CAC

Trp Val Lys Gln Asn Pro Glu Gln Gly Leu
TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG

Glu Trp Ile Gly Tyr Phe Ser Pro Gly Asn
GAA TGG ATT GGA TAT TTT TCT CCC GGA AAT

Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys
GAT GAT TTT AAA TAC AAT GAG AGG TTC AAG

Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser
GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC

Ser Ser Thr Ala Tyr Val Gln Leu Asn Ser
TCC AGC ACT GCC TAC GTG CAG CTC AAC AGC

Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe
CTG ACA TCT GAG GAT TCT GCA GTG TAT TTC

Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp
TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG
                              CC49 VH ------>
Gly Gln Gly Thr Ser Val Thr Val Ser Ser
GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA
```

FIG. 26 (CONT.)

***Nhe* I**

TAA AAA**GCTAGC**G ATGAATCCGT CAAAACATCA

***Bcl* I**

TCTTACATAA AGTCACTTGG **TGATCA**AGCT

CATATCATTG TCCGGCAATG GTGTGGGCTT

TTTTTGTTTT CTATCTTTAA AGATCATGTG

AAGGAAAAAA CGGGAAAATC GGTCTGCGGG

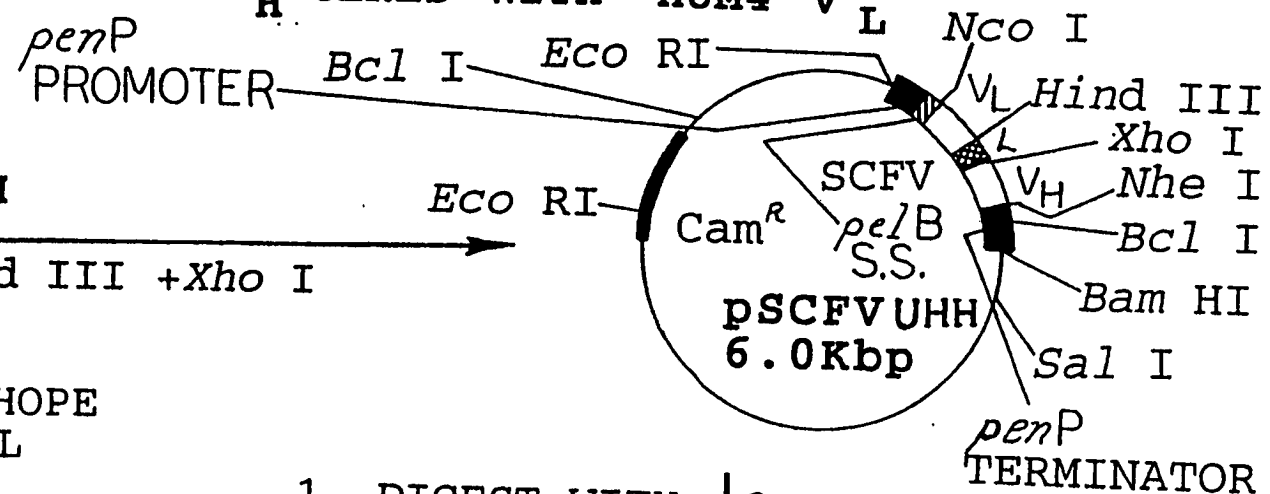
AAAGGACCGG GTTTTTGTCG AAATCATAGG

***Bam* HI**

CGAATGGGTT GGATTGTGAC AAAATTC**GGA TCC**

FIG. 27

CONSTRUCTION OF PLASMID pSCFV UHH. GENERATION OF A COMBINATORIAL LIBRARY OF V_H GENES WITH HUM4 V_L



pSCFV UNIH

1. DIGEST WITH *Hind* III +*Xho* I PURIFY AWAY L
2. LIGATE WITH UNIHOPE *Hind* III/*Xho* I L

1. DIGEST WITH *Xho* I+*Nhe* I. PURIFY AWAY CC49 V_H
2. LIGATE WITH HUMAN V_H *Xho* I/*Nhe* I PCR DNA INSERTS

COMBINATORIAL LIBRARY OF HUMAN V_H GENES WITH HUMAN SUBGROUP IV V_L EXPRESSED AS SCFV PRODUCTS

FIG. 28

```
                                    Eco RI
CTCATGTTTG ACAGCTTATC ATCGATGAAT

TCCATCACTT CCCTCCGTTC ATTTGTCCCC

GGTGGAAACG AGGTCATCAT TTCCTTCCGA

AAAAACGGTT GCATTTAAAT CTTACATATG

TAATACTTTC AAAGACTACA TTTGTAAGAT

TTGATGTTTG AGTCGGCTGA AAGATCGTAC

GTACCAATTA TTGTTTCGTG ATTGTTCAAG

CCATAACACT GTAGGGATAG TGGAAAGAGT

GCTTCATCTG GTTACGATCA ATCAAATATT

                         |<--- pelB Signal
                         |Met Lys Tyr Leu
CAAACGGAGG GAGACGATTT TG |ATG AAA TAC CTA
Sequence
Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu
TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA
                   Nco I --->|<--- H4VL
Leu Ala Ala Gln Pro Ala Met Ala|Asp Ile
CTC GCT GCC CAA CCA GCC ATG GCC|GAC ATC

Val Met Thr Gln Ser Pro Asp Ser Leu Ala
GTG ATG ACC CAG TCT CCA GAC TCC CTG GCT

Val Ser Leu Gly Glu Arg Ala Thr Ile Asn
GTG TCT CTG GGC GAG AGG GCC ACC ATC AAC

Cys Lys Ser Ser Gln Ser Val Leu Tyr Ser
TGC AAG TCC AGC CAG AGT GTT TTA TAC AGC
```

FIG. 28 (CONT.)

Ser Asn Asn Lys Asn Tyr Leu Ala Trp Tyr
TCC AAC AAT AAG AAC TAC TTA GCT TGG TAC

Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu
CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG

Leu Ile Tyr Trp Ala Ser Thr Arg Glu Ser
CTC ATT TAC TGG GCA TCT ACC CGG GAA TCC

Gly Val Pro Asp Arg Phe Ser Gly Ser Gly
GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG

Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC

Ser Leu Gln Ala Glu Asp Val Ala Val Tyr
AGC CTG CAG GCT GAA GAT GTG GCA GTT TAT

Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu
TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC

$H4V_L$

Thr Phe Gly Gly Gly Thr Lys Val Val Ile
ACT TTC GGC GGA GGG ACC AAG GTG GTG ATC

***Hind* III LINKER**

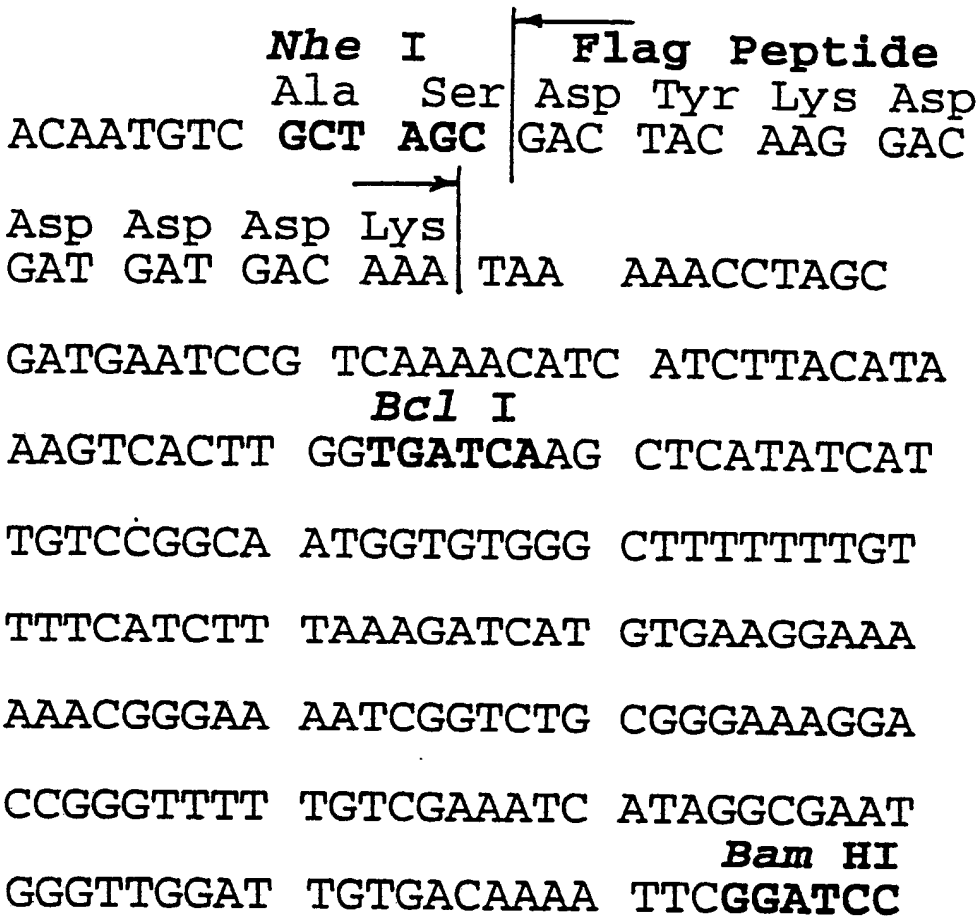
Lys | Leu Ser Ala Asp Asp Ala Lys Lys Asp
AAG | **CTT** AGT GCG GAC GAT GCG AAA AAG GAT

Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp
GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC

LINKER *Xho* I

Asp Ala Lys Lys Asp Leu | Glu
GAT GCT AAA AAG GAC **CTC** | **GAG**

FIG. 28 (CONT.)


Nhe I
Flag Peptide
Ala Ser Asp Tyr Lys Asp
ACAATGTC GCT AGC GAC TAC AAG GAC
Asp Asp Asp Lys
GAT GAT GAC AAA TAA AAACCTAGC
GATGAATCCG TCAAAACATC ATCTTACATA
Bcl I
AAGTCACTT GGTGATCAAG CTCATATCAT
TGTCCGGCA ATGGTGTGGG CTTTTTTTGT
TTTCATCTT TAAAGATCAT GTGAAGGAAA
AAACGGGAA AATCGGTCTG CGGGAAAGGA
CCGGGTTTT TGTCGAAATC ATAGGCGAAT
Bam HI
GGGTTGGAT TGTGACAAAA TTCGGATCC

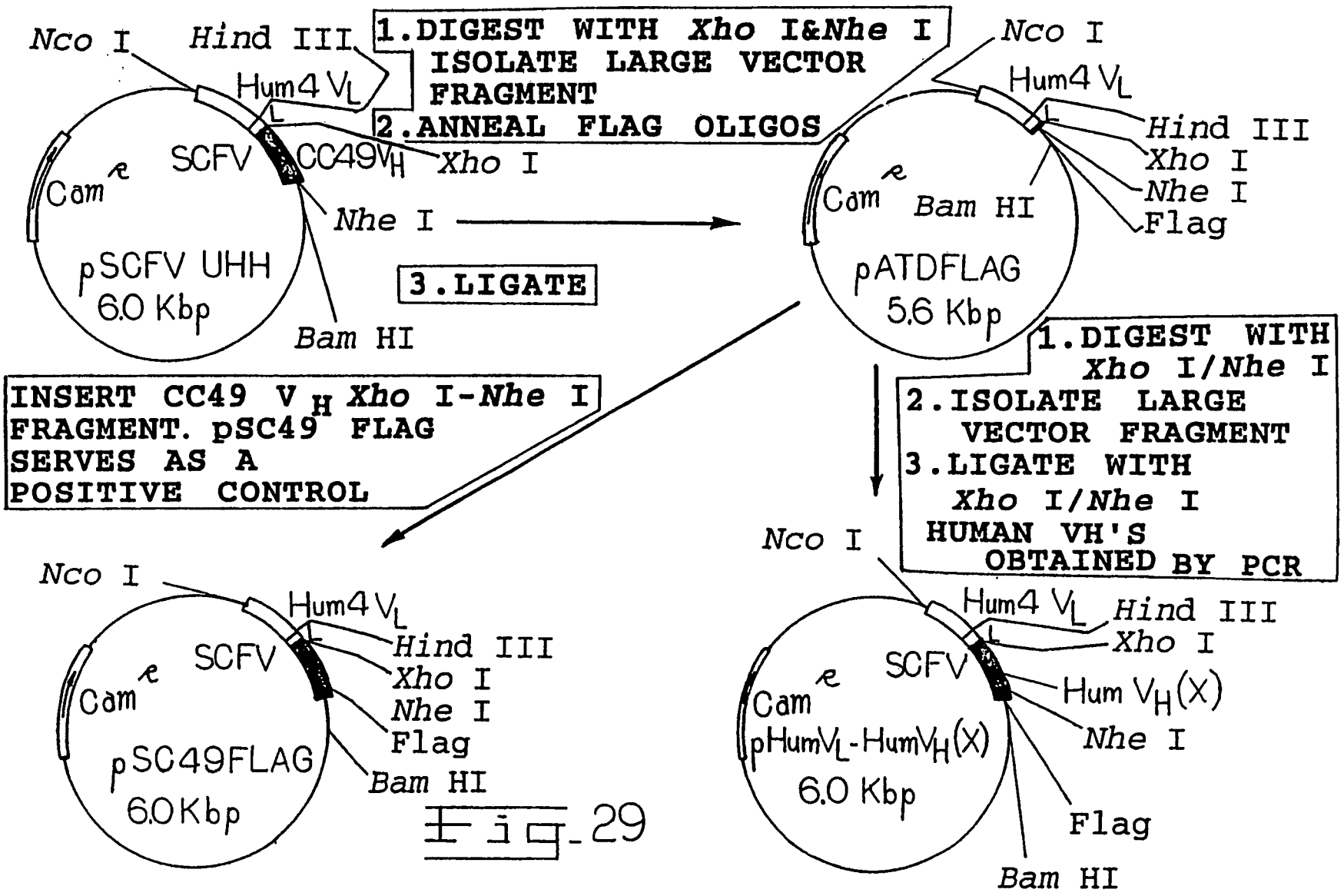

Nco I
Hind III
1.DIGEST WITH Xho I&Nhe I
ISOLATE LARGE VECTOR FRAGMENT
2.ANNEAL FLAG OLIGOS
Hum4 VL
SCFV
CC49VH
Xho I
Cam
Nhe I
pSCFV UHH
6.0 Kbp
Bam HI
3.LIGATE
INSERT CC49 VH Xho I-Nhe I FRAGMENT. pSC49 FLAG SERVES AS A POSITIVE CONTROL
Nco I
Hum4 VL
Hind III
Xho I
Nhe I
Cam
Bam HI
pATDFLAG
5.6 Kbp
Flag
1.DIGEST WITH Xho I/Nhe I
2.ISOLATE LARGE VECTOR FRAGMENT
3.LIGATE WITH Xho I/Nhe I HUMAN VH'S OBTAINED BY PCR
Nco I
Hum4 VL
Hind III
Xho I
SCFV
Cam
Hum VH(X)
Nhe I
pHumVL-HumVH(X)
6.0 Kbp
Flag
Bam HI
Nco I
Hum4 VL
Hind III
Xho I
Nhe I
Flag
SCFV
Cam
pSC49FLAG
6.0Kbp
Bam HI

Fig. 29

FIG. 30

Eco **RI**

CTCATGTTTG ACAGCTTATC ATCGAT**GAAT**

TCCATCACTT CCCTCCGTTC ATTTGTCCCC

GGTGGAAACG AGGTCATCAT TTCCTTCCGA

AAAAACGGTT GCATTTAAAT CTTACATATG

TAATACTTTC AAAGACTACA TTTGTAAGAT

TTGATGTTTG AGTCGGCTGA AAGATCGTAC

GTACCAATTA TTGTTTCGTG ATTGTTCAAG

CCATAACACT GTAGGGATAG TGGAAAGAGT

GCTTCATCTG GTTACGATCA ATCAAATATT

***pelB* Signal**

Met	Lys	Tyr	Leu
ATG	AAA	TAC	CTA

CAAACGGAGG GAGACGATTT TG

Sequence

Leu	Pro	Thr	Ala	Ala	Ala	Gly	Leu	Leu	Leu
TTG	CCT	ACG	GCA	GCC	GCT	GGA	TTG	TTA	TTA

***Nco* I** → ← **$H4V_L$**

Leu	Ala	Ala	Gln	Pro	Ala	Met	Ala	Asp	Ile
CTC	GCT	GCC	CAA	CCA	**GCC**	**ATG**	**GCC**	GAC	ATC

Val	Met	Thr	Gln	Ser	Pro	Asp	Ser	Leu	Ala
GTG	ATG	ACC	CAG	TCT	CCA	GAC	TCC	CTG	GCT

Val	Ser	Leu	Gly	Glu	Arg	Ala	Thr	Ile	Asn
GTG	TCT	CTG	GGC	GAG	AGG	GCC	ACC	ATC	AAC

Cys	Lys	Ser	Ser	Gln	Ser	Val	Leu	Tyr	Ser
TGC	AAG	TCC	AGC	CAG	AGT	GTT	TTA	TAC	AGC

FIG. 30 (CONT.)

Ser Asn Asn Lys Asn Tyr Leu Ala Trp Tyr
TCC AAC AAT AAG AAC TAC TTA GCT TGG TAC

Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu
CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG

Leu Ile Tyr Trp Ala Ser Thr Arg Glu Ser
CTC ATT TAC TGG GCA TCT ACC CGG GAA TCC

Gly Val Pro Asp Arg Phe Ser Gly Ser Gly
GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG

Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC

Ser Leu Gln Ala Glu Asp Val Ala Val Tyr
AGC CTG CAG GCT GAA GAT GTG GCA GTT TAT

Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu
TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC

H4V$_L$

Thr Phe Gly Gly Gly Thr Lys Val Val Ile
ACT TTC GGC GGA GGG ACC AAG GTG GTG ATC

***Hind* III LINKER**

Lys | Leu Ser Ala Asp Asp Ala Lys Lys Asp
AAG | **CTT** AGT GCG GAC GAT GCG AAA AAG GAT

Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp
GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC

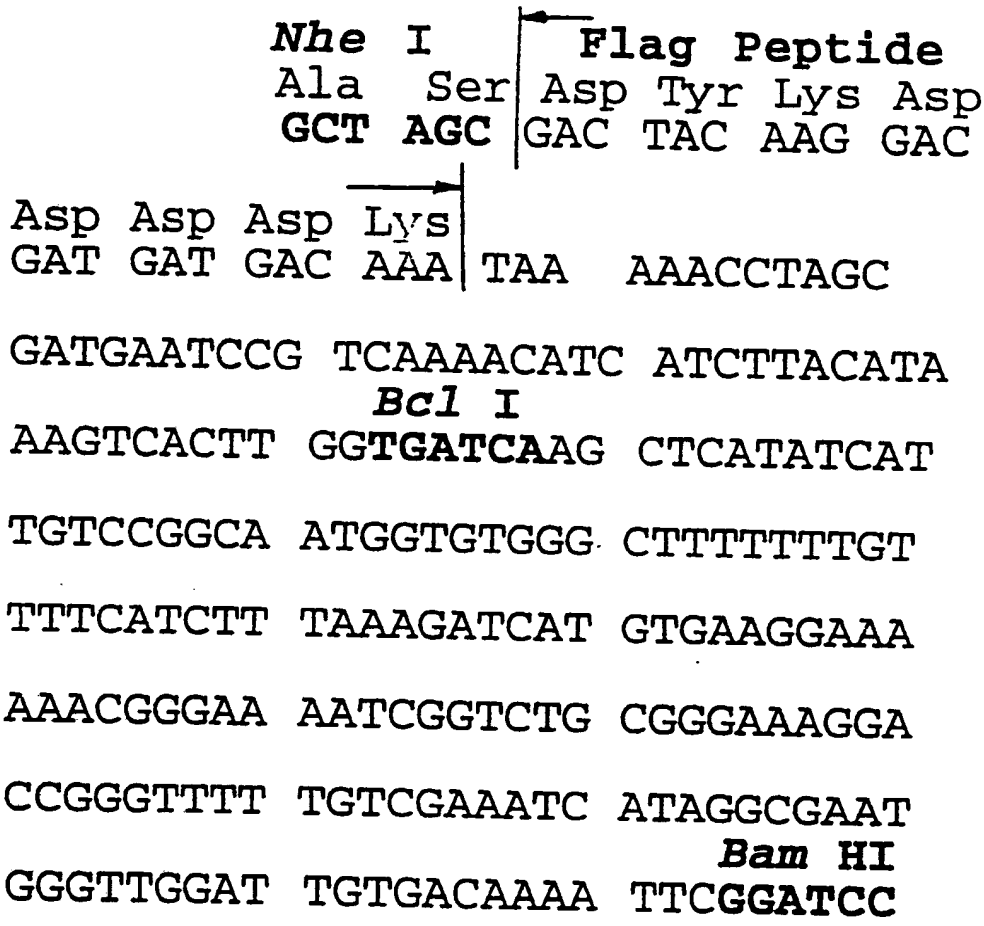
LINKER ***Xho* I** **CC49 V$_H$**

Asp Ala Lys Lys Asp Leu | Glu Val Gln Leu
GAT GCT AAA AAG GAC **CTC** | **GAG** GTT CAG TTG

FIG. 30 (CONT.)

```
Gln Gln Ser Ala Glu Leu Val Lys Pro Gly
CAG CAG TCT GCT GAG TTG GTG AAA CCT GGG

Ala Ser Val Lys Ile Ser Cys Lys Ala Ser
GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT

Gly Tyr Thr Phe Thr Asp His Ala Ile His
GGC TAC ACC TTC ACT GAC CAT GCA ATT CAC

Trp Val Lys Gln Asn Pro Glu Gln Gly Leu
TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG

Glu Trp Ile Gly Tyr Phe Ser Pro Gly Asn
GAA TGG ATT GGA TAT TTT TCT CCC GGA AAT

Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys
GAT GAT TTT AAA TAC AAT GAG AGG TTC AAG

Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser
GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC

Ser Ser Thr Ala Tyr Val Gln Leu Asn Ser
TCC AGC ACT GCC TAC GTG CAG CTC AAC AGC

Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe
CTG ACA TCT GAG GAT TCT GCA GTG TAT TTC

Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp
TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG
                                CC49 VH ------>
Gly Gln Gly Thr Ser Val Thr Val Ser Ser
GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA
```

FIG. 30 (CONT.)


Nhe I
Flag Peptide
Ala Ser Asp Tyr Lys Asp
GCT AGC GAC TAC AAG GAC
Asp Asp Asp Lys
GAT GAT GAC AAA TAA AAACCTAGC
GATGAATCCG TCAAAACATC ATCTTACATA
Bcl I
AAGTCACTT GGTGATCAAG CTCATATCAT
TGTCCGGCA ATGGTGTGGG CTTTTTTTGT
TTTCATCTT TAAAGATCAT GTGAAGGAAA
AAACGGGAA AATCGGTCTG CGGGAAAGGA
CCGGGTTTT TGTCGAAATC ATAGGCGAAT
Bam HI
GGGTTGGAT TGTGACAAAA TTCGGATCC

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 91/00583

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member		
AU 89/43540	AU 44299/89	BR 8907126	CA 2000913
	DK 1499/90	EP 365997	EP 397821
	FI 903056	HU 896255	IL 92037
	JP 3502889	NO 902696	WO 9004410
	ZA 8907858		
AU 89/44299	AU 43540/89	BR 8907126	CA 2000913
	DK 1499/90	EP 397821	EP 365997
	FI 903056	HU 896255	HU 56878
	IL 92037	JP 3502889	NO 902696
	WO 9004410	ZA 8907858	

International Application No: PCT/ AU91 /00583

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 46 line 3 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☐

Name of depositary institution

American Type Culture Collection

Address of depositary institution (including postal code and country)

12301 Parklawn Drive,
Rockville, Maryland 20852 USA

Date of deposit	Accession Number
April 18, 1990	ATCC HB 10426

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (If the indications are not for all designated States)

The cell lines will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a United States Patent is issued citing the cell lines.

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

In accordance with Regulation 3.25 of the Patents Regulations (Australia Statutory Rules 1991 No 71), samples of materials deposited in accordance with the Budapest Treaty in relation to an Australian Patent Request are only to be provided before: the patent is granted on the application; or the application has lapsed or been withdrawn or refused; to a person who is: a skilled addressee without an interest in the invention; and nominated by a person who makes a request for the furnishing of those samples.

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☒ The date of receipt (from the applicant) by the International Bureau is

27 JAN 1992

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ AU91 / 00583

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 46 line 3 of the description

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E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☒ The date of receipt (from the applicant) by the International Bureau is

27 JAN 1992

(Authorized Officer)

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent classification (IPC) or to both National Classification and IPC

Int. Cl.[5] C12N 15/13, C12N 5/10, A61K 39/395, 47/48, 49/02, 43/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	WPAT, Chemical Abstracts, Derwent Databases: Keywords: TAG 72 or (tumor or tumour) and associated and glyco:

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU Search: C12N 15/13, C12P 21/08 Biotechnology Abstracts Derwent Database: Keywords: TAG 72 or (tumor or tumour) and associated and glyco:

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category*	Citation of Document, [11] with indication, where appropriate of the relevant passages [12]	Relevant to Claim No [13]
X	AU,A, 43540/89 (THE DOW CHEMICAL COMPANY) 26 April 1990 (26.04.90). See claims 1, 2, 5-9, 11-14, 16, 27, 29, 30, 32-35, 37-39	1-33
X	AU,A, 44299/89 (THE DOW CHEMICAL COMPANY) 14 May 1990 (14.05.90). See claims 1, 7-11, 13-18, 29, 31-38, 48-50	1-3, 5-33
Y	Whittle, N et al: 'Expression in COS cells of a mouse-human chimaeric B72.3 antibody'. Protein Engineering, volume 1, no. 6, pages 499-505, 1987	1, 6-9
Y	Brady, R L et al.: "Crystallization and Preliminary X-ray Diffraction Study of a Chimaeric Fab' Fragment of Antibody (continued)	1, 6-9

* Special categories of cited documents : [10]

"A" Document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior t the international filing date but later than the priority date claimed

"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 July 1992 (30.07.92)	21 Aug 1992 (21.08.92)
International Searching Authority	Signature of Authorized Officer
AUSTRALIAN PATENT OFFICE	K AYERS Kara Ayers

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Binding Tumour Cells". J Mol Biol, 219, pages 603-604, 1991

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4a

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.